TOLL-LIKE RECEPTORS AND MACROPHAGE POLARIZATION IN THE LOOSENING OF TOTAL HIP REPLACEMENTS

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Contents

1. List of original publications

This thesis is based on the following original publications. The studies are referred to in the text by their Roman numerals I-IV.

I. Lähdeoja T, **Pajarinen J**, Kouri VP, Sillat T, Salo J, Konttinen YT. Toll-like receptors and aseptic loosening of hip endoprosthesis-a potential to respond against danger signals? J Orthop Res 2010;28:184-90.

II. **Pajarinen J**, Cenni E, Savarino L, Gomez-Barrena E, Tamaki Y, Takagi M, Salo J, Konttinen YT. Profile of toll-like receptor-positive cells in septic and aseptic loosening of total hip arthroplasty implants. J Biomed Mater Res A 2010;94:84-92.

III. **Pajarinen J**, Mackiewicz Z, Pöllänen R, Takagi M, Epstein NJ, Ma T, Goodman SB, Konttinen YT. Titanium particles modulate expression of Toll-like receptor proteins. J Biomed Mater Res A 2010;92:1528-37.

IV. **Pajarinen J**, Kouri VP, Jämsen E, Li TF, Mandelin J, Konttinen YT. Macrophages response to wear particles is determined by macrophage polarization (submitted)

In addition, some unpublished results are presented.

2. Abbreviations

ACTB	β-actin
AP-1	activator protein 1
BSA	bovine serum albumin
CCL	chemokine (C-C motif) ligand
CD	cluster of differentiation
cDNA	complementary DNA
CTSK	cathepsin k
CXCL	chemokine (C-X-C motif) ligand
CX3CL	chemokine (C-X3-C motif) ligand
DAMP	danger-associated molecular pattern
DNA	deoxyribonucleic acid
ECM	extracellular matrix
EFG	epidermal growth factor
FBS	fetal bovine serum
Fc	fold change
FGF	fibroblast growth factor
FLT	fms-like tyrosine kinase
GAPDH	glyceraldehyde 3-phosphate dehydrogenase
G-CSF	granulocyte colony-stimulating factor
GM-CSF	granulocyte-macrophage colony-stimulating factor
GO	gene ontology
HMGB1	high-mobility group box 1
HSP	heat-shock protein
HXLPE	highly cross-linked polyethylene
IFN	interferon
IL	interleukin
IL-1ra	interleukin-1 receptor antagonist
IRF	interferon regulatory factor
LPS	lipopolysaccharide
LTA	lipoteichoic acid
MAL	MyD88 adaptor-like
MAPK	mitogen-activated protein kinases
M-CSF	macrophage colony-stimulating factor
MHC	major histocompatibility complex
MMP	matrix metalloproteinase
MyD88	myeloid differentiation factor 88
NFAT	nuclear factor of activated T cells
NF-κB	nuclear factor kappa-light-chain-enhancer of activated B cells
NE	neutrophil elastase
OPG	osteoprotegerin
OSM	oncostatin M
PAMP	pathogen-associated molecular pattern
PBGD	porphobilinogen deaminase
PBMC	peripheral blood mononuclear cells

PBS	phosphate-buffered saline
PGE2	prostaglandin E2
PI3K	phosphoinositide 3-kinase
PMMA	polymethyl methacrylate
PPARγ	peroxisome proliferator-activated receptor-γ
PRR	pattern recognition receptor
qRT-PCR	quantitative real-time polymerase chain reaction
RANK	receptor activator of nuclear factor kappa B
RANKL	receptor activator of nuclear factor kappa B ligand
RNA	ribonucleic acid
RPLP0	large ribosomal protein P0
sCD40L	soluble cluster of differentiation 40 ligand
sIL-2rα	soluble interleukin-2 receptor alpha
SPIA	signaling pathway impact analysis
STAT	signal transducer and activator of transcription
TGF	transforming growth factor
Th	T helper cell
THR	total hip replacement
TIR	toll-interleukin 1 receptor
TLR	toll-like receptor
TNF	tumor necrosis factor
TNFSF	tumor necrosis factor super family
TRAM	TRIF-related adaptor molecule
TRAP	tartrate-resistant acidic phosphatase
TRIF	TIR domain–containing adaptor inducing interferon-β
UHMWPE	ultra high molecular weight polyethylene
VEGF	vascular endothelial growth factor

3. Abstract

Treatment of patients suffering from end-stage hip arthritis was revolutionized in the late 1950s by the development of modern total hip replacement (THR). This operation effectively alleviates pain and improves function of these patients; unsurprisingly, the demand for THR surgery has been rapidly increasing. Despite the fact that the clinical outcome of THR surgery is typically excellent and that THR is generally considered one of the most successful surgical interventions, THR is not without complications. Its two main complications are implant infection, also known as septic loosening, and long-term aseptic implant loosening. In both cases, the bone originally surrounding the implant is resorbed, and the implant loosens, necessitating revision operations which are costly, technically demanding, and are causes of morbidity and even mortality. Despite new, more durable implant materials and advances in surgical technique, the THR revision numbers have been on a steady increase, closely following the increase in primary THR operation numbers and widening of the primary operation indications to include younger and more active patients.

Traditionally, septic and aseptic joint replacement loosening has been considered two separate entities, in which septic loosening is due to chronic inflammation caused by bacterial infection of implant components, whereas aseptic loosening is driven by a foreign body reaction against about 1-µm biomaterial wear particles that are generated due to abrasion between THR components. According to this particle-disease hypothesis, these wear particles are released into peri-implant tissues where they are phagocytosed by macrophages. The macrophages respond to this foreign material by producing an array of inflammatory mediators causing the local micro-environment to favor formation of osteoclasts and active bone resorption which ultimately leads into implant loosening. The fundamental events in the pathogenesis of aseptic loosening thus are wear-particle recognition, phagocytosis, and subsequent macrophage activation to the inflammatory phenotype. The exact mechanisms by which biomaterial particles are recognized by macrophages and how their phagocytosis leads to macrophage activation have, however, remained elusive, as has the fundamental question of why implant loosening develops in only a relatively small proportion of patients, even though all hip replacements generate wear particles.

During the last decade, the strict dichotomy between septic and aseptic loosening has been increasingly questioned. For instance, subclinical bacterial biofilms occur in at least in some cases of apparently aseptic impant loosening. Likewise, pro-inflammatory and osteolytic properties of wear particles depend at least partially on the presence of bacterial components adhering to their surfaces. These observations lead to the hypothesis that recognition of bacterial product-coated wear particles and subsequent activation of interface tissue macrophages into the inflammatory phenotype might be mediated by macrophages Toll-like receptors (TLRs).

TLRs are a family of pattern recognition receptors that recognize various exo- and endogenous danger-signal molecules and mediate macrophage activation. We evaluated the extent to which the various TLRs occur and how they localize in the aseptic interface tissues by using immunohistochemistry and qRT-PCR. The direct effect of wear particles on TLR levels we then evaluated in an animal model of wear-particle-induced inflammation and in a macrophage culture system. In hopes of identifying cell populations potentially useful as diagnostic markers of subclinical implant-related infection, cell

populations occurring in typical septic and aseptic interface tissue were evaluated by use of cell-type specific antibodies and immunohistochemistry.

Macrophages and foreign body giant cells of aseptic interface tissue expressed TLR1-9 and actively produced all of these, except TLR3 and TLR7. Based on the literature it seems likely that TLRs, especially TLR2, TLR2/1, TLR2/6, TLR4, and TLR9, are involved in wear-particle recognition and subsequent macrophage activation either by directly binding to the particles' polymeric surfaces or to metal ions released from the implant but probably more likely via recognition of exo- or endogenous danger signal molecules adhering to particle surfaces. In a mouse model of particle-induced inflammation, wear particles led to down-regulation rather than to up-regulation of TLR levels, and had no effect on TLR mRNA levels in the mouse macrophage culture system. Up-regulation of TLRs in aseptic interface tissue is thus likely mediated by factors other than a direct effect of wear particles on macrophages. Comparison of inflammatory cell populations present in septic and aseptic interface tissues revealed that, in addition to neutrophils, B lymphocytes and plasma cells might serve as useful marker cells in the diagnosis of low-grade implant-related infection.

Macrophages are a functionally dynamic and adaptive population of cells that can assume various functional phenotypes as guided by signals from the local micro-environment. Differing macrophage phenotypes express various levels of TLRs and other pattern-recognition receptors and have fundamentally differing abilities to produce pro-inflammatory and chemotactic mediators. We thus further hypothesized that this macrophage phenotype, or macrophage polarization, may be an important determinant of the way that macrophages react to wear particles. To test this hypothesis, we differentiated M0, M1, and M2 macrophages from human monocytes and compared their responses to titanium-particle stimulus using genome-wide microarray analysis and a multiplex cytokine assay.

In comparison to non-activated M0 macrophages, the overall chemotactic and inflammatory responses to wear particles was greatly enhanced in M1 macrophages and effectively suppressed in M2 macrophages, which effectively contained particles in intracellular compartment. Results suggest that in addition to wear-particle characteristics and biomolecules adhering to particle surfaces, the local cytokine milieu also determines the extent to which macrophages are activated by wear particles.This effect may have been due to differing expression of TLRs or other pattern-recognition receptors between these macrophage types. Limiting the action of M1-polarizing factors such as bacterial biofilm formation in the interface tissue and perhaps also promoting M2 macrophage polarization by biomaterial solutions or pharmacologically might thus limit osteolysis resulting from inevitably forming wear particles. Interesting is also the emerging hypothesis that the low-grade inflammation at systemic level associated, for example, with obesity and atherosclerosis, might be a determinant in the general systemic M1-M2 balance of macrophages and thus in the susceptibility of an individual to develop aseptic osteolysis.

4. Introduction

Since its development in the late 1950s, total hip replacement (THR) has been a successful treatment for patients suffering from end-stage hip joint arthritis. The clinical outcomes of the operation are generally excellent and the demand for THR surgery has been rapidly increasing (Ethgen et al 2004, OECD Health data 2011, Skyttä et al 2011). The two main complications of THR are implant infection, also known as septic loosening, and aseptic loosening. In both cases, the bone originally surrounding the implant is resorbed, and the implant loosens, necessitating technically demanding revision operations. Despite the advances in implant materials and in surgical techniques, THR revision numbers have been on a steady increase (Kurtz et al 2005, Bozic et al 2009, National agency for Medicines 2009).

Traditionally, septic and aseptic prosthesis loosening has been considered two separate entities, in which septic loosening is due to chronic inflammation caused by bacterial infection of joint replacement components (Zimmerli et al 2004). In contrast, aseptic loosening is driven by a foreign body reaction against biomaterial wear particles that are generated due to abrasion between THR components (Goodman et al 2009, Gallo et al 2012). These wear particles are in the peri-implant tissues phagocytosed by macrophages which are induced to produce an array of inflammatory mediators causing the local micro-environment to favor formation of osteoclasts and active bone resorption (Ingham and Fisher 2005, Konttinen et al 2005). Wear-particle-induced macrophage activation to the inflammatory phenotype is thus considered a fundamental event in the pathogenesis of aseptic loosening but the exact mechanisms of this wear-particle-induced macrophage activation have remained elusive.

Toll-like receptors (TLRs) are a family of ten pattern recognition receptors which recognize various pathogen-derived molecules such as lipoteichoic acid (LTA) and lipopolysaccharide (LPS, Kawai and Akira 2010). In addition, endogenous TLR ligands, released because of cell necrosis and extracellular matrix (ECM) damage, appear to exist (Bianchi 2007, Kono and Rock 2008). Collectively, these TLR ligands are known as danger-associated molecular patterns (DAMP); recognition of a DAMP molecule by TLR and subsequent TLR signaling mediates macrophage activation and production of inflammatory mediators (Gordon 2003).

In addition to this TLR-mediated macrophage activation, macrophages can assume distinct activation phenotypes as a response to the local cytokine microenvironment (Ma et al 2003, Mosser and Edwards 2008). Reflecting the concept and following the nomenclature of CD4+ T helper (Th) lymphocyte polarization into Th1 and Th2 cells, a corresponding concept has been established of functional macrophage polarization into M1 and M2 macrophages, or “classically” and “alternatively” activated macrophages. M1 macrophages are an inflammatory macrophage phenotype that is especially related to Th1 response and immunity against intracellular pathogens, whereas M2 macrophages are related to a wide range of physiological and pathological processes such as allergy, parasite immunity, tissue healing, homeostasis, and fibrosis (Schroder et al 2004, Martinez et al 2009).

During the last decade, the strict dichotomy between septic and aseptic joint replacement loosening has been increasingly questioned (Nelson et al 2005). For example, recent observations are that wear particles are relatively inert, cause only limited macrophage activation and osteolysis, and have their inflammatory properties largely dependent on bacterial structural components adhering to their surfaces (Greenfield et al

2005). These observations lead to the hypothesis that recognition of DAMP-coated wear particles and following activation of interface tissue macrophages into the inflammatory phenotype might be mediated by macrophages' TLRs. Additionally, as M1 and M2 macrophages express different levels of TLRs and have dissimilar abilities to produce inflammatory mediators, we further hypothesized that macrophage polarization might be an important determinant in the way that macrophages react to wear particles.

The present study aimed to evaluate the possible presence and localization of various TLRs in the aseptic interface tissue and to discover whether wear particles directly regulate TLR levels in a mouse model of wear-particle-induced inflammation and in a macrophage culture system. In hopes of identifying cell populations potentially useful as diagnostic markers of subclinical implant-related infection, we characterized cell populations occurring in typical septic and aseptic interface tissue. Additionally, the aim was to discover whether macrophage polarization affects macrophage wear-particle responses.

5. Review of the literature

5.1. Total hip replacement

5.1.1. Hip replacement designs and materials

Treatment of patients suffering from end-stage hip arthritis was revolutionized in the late 1950s and early 1960s by the development of modern THR by British orthopedic surgeon Sir John Charnley. The original Charnley THR was composed of a stainless-steel femoral component and an acetabular cup made of ulta high molecular-weight polyethylene (UHMWPE), that formed a low-friction and relatively durable articulation (Charnley 1961). Hip replacement components were firmly fixed to the surrounding bone with polymethyl methacrylate (PMMA, bone cement). Charney's THR design was superior to other contemporary hip replacement solutions, which failed primarily due to poor design and unfortunate material choices; his THR design rapidly gained popularity (Learmonth et al 2007). Since then, some major improvements to original Charnley hip replacement design (such as more wear-resistant implant materials) and surgical techniques (such as modern cementing techniques) have been introduced and new hip replacement designs (such as uncemented THR) emerged. The basic principles of Charnley "low-frictional torque arthroplasty" have, however, remained largely the same for 50 years (Charnley 1970, Learmonth et al 2007).

A typical modern THR implant is composed of three separate parts: a cup, a head, and a stem. The head is attached to the femoral intramedullary stem, and the cup and the head articulate in a ball-and-socket fashion closely mimicking the normal hip joint anatomy (Figure 1). The cup-part is either a single component or a composite of two separate parts, an outer metal shell facing the bony acetabulum and an inner liner forming the actual articulating surface of the implant (modular cup). The femoral component is also typically modular so that the intramedullary stem and the articulating head of the joint are separate components, possibly made of different materials. During the THR operation, the femoral head and part of the femoral neck are removed and the acetabulum and femoral medullary canal reamed and cleaned. The cup of the THR implant is then inserted into the acetabulum and the stem into the reamed medullary canal of the femur. Fixation of the components to the surrounding bone is achieved either with PMMA (cemented implants) or by friction and the eventual growth of bone tissue directly onto the implants surface (uncemented implants). Based on mode of fixation, THR implants are classified into these two broad categories: cemented and uncemented.

In cemented implants, fixation to bone is achieved by an additional layer of bone cement placed between the bone and the implant (Charnley 1964, Scheerlinck et al 2006, Hernigou et al 2009). If appropriately inserted under constant pressure, PMMA effectively seeps between the trabeculae of endosteal spongy bone and functions as a mechanical interlock between hip replacement components and bone. Cemented hip replacement stems are further divided into two fundamentally different design categories depending on mode of attachment to bone cement, namely a highly polished taper-slip / force-closed design and a composite beam / shape-closed design (Scheerlinck et al 2006). The idea for the former is that there is no firm adhesion between hip replacement and PMMA mantle and that the femoral stem is firmly pressed, and even is slowly forced to migrate, deeper into the PMMA mantle and femoral canal by continuous axial loading. The composite beam-design, in contrast, aims at firm attachment of the implant to the PMMA mantle via surface roughening and modifications.

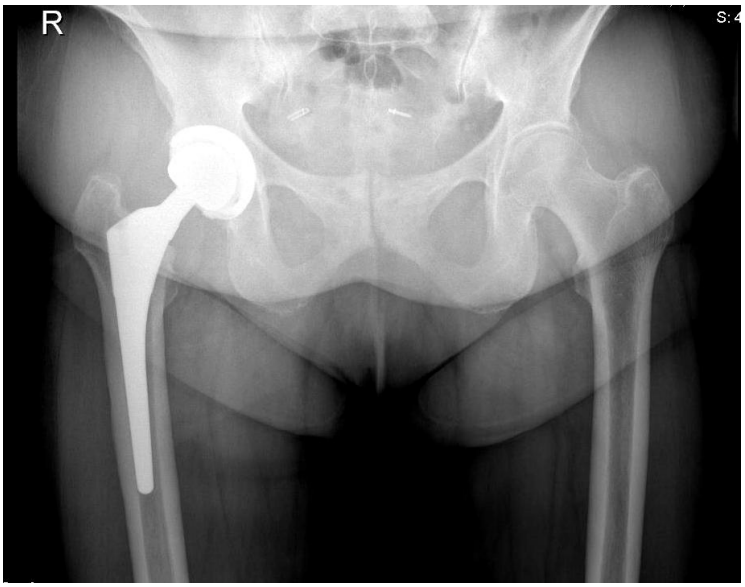

Figure 1. An uncemented total hip replacement composed of three parts: a stem, a head, and a modular cup. The head is attached to the intramedullary femoral stem. The rounded head articulates with the acetabular cup in a ball-and-socket fashion closely mimicking normal hip joint anatomy. The implant is well fixed to the surrounding bone with no signs of osteolysis.

In uncemented hip replacements, fixation is achieved initially by mere friction between implant components and the bone bed (Learmonth et al 2007). In this “press fit” or “friction fit” technique, the slightly over-sized implant components are tightly fitted into their corresponding bony sockets to achieve firm mechanical locking to the bone. Additionally, the surfaces of uncemented hip replacements are typically trabecular or porous; a firm secondary stabilization of the implant components occurs over time as bone grows directly onto the micro- or macroporous implant surface, or in case of a tantalum surface, even into the trabecular surface itself. In some uncemented THR designs, the porous surface is further coated with osteoconductive material such as hydroxylapatite.

THR implants can be further classified based on the material of their bearing surfaces (Learmonth et al 2007). Following the principles of the original Charnley hip replacement design, the stainless-steel head on an UHMWPE cup was the gold standard for a bearing surface for several decades. However, since recognition that extensive wear of the UHMWPE acetabular cup is the leading cause of osteolysis and aseptic loosening, more wear-resistant bearing surfaces have been vigorously sought.

During the last decade, this research has led to development of first and second generation highly cross-linked polyethylene (HXLPE) plastics produced by gamma irradiation of UHMWPE. Compared to UHMWPE, HXLPE acetabular liners have greatly reduced wear rates; accordingly, traditional UHMWPE, although still used in some hip replacement designs, has been largely replaced by HXPLE as the material of choice for acetabular liners (Muratoglu et al 2001).

Stainless steel is a still widely used material for the femoral head and stem for cemented hip replacements, although alternative metal alloys have also emerged, such as cobalt-chromium-molybdenum. Titanium alloy, composed typically of titanium, aluminum, and vanadium, is the usual material of choice for femoral stems and the outer

shell of the acetabular cup in uncemented hip replacements, but due to its relative softness and low wear resistance, it is not suited for use as a bearing surface.

The search for more wear-resistant bearing surfaces has also led to the development of metal-on-metal (typically various cobalt-chromium alloys), ceramic-on-ceramic (typically alumina or zirconia ceramics), and ceramic-on-HXLPE bearings, which have greatly reduced wear rates in comparison to traditional metal-on-UHMWPE bearings but which present other possible problems, such as fractures of brittle ceramic components (Jeffers and Walter 2012). In particular; the surprisingly high failure rates and the release of high and systemically detectable levels of metal ions and nano-sized metal particles from meta-on-metal implants, has recently led to much concern (Zywiel et al 2011, Cohen 2012, Smith et al 2012).

New, still more wear-resistant and biocompatible bearing-surface materials, such as amorphous diamond are being developed but have not yet reached clinical use (Alakoski et al 2008).

5.1.2. Indications for THR operation

About 60 to 80% of THR operations are performed for primary osteoarthritis of the hip joint and 5 to 10% for rheumatoid arthritis (Havelin et al 2000, Lucht 2000, Furnes et al 2001, Puolakka et al 2001, Pedersen et al 2005, National agency for Medicines 2009, Fevang et al 2010, Hailer et al 2010). Other less-common indications for THR surgery include trauma, tumors, and secondary osteoarthritis with various causes such as avascular necrosis of the femoral head or developmental dysplasia of the hip. The number of THRs performed for rheumatoid arthritis has been decreasing, likely due to the increased effectiveness of immunosuppressive therapies, whereas operation numbers for osteoarthritis have shown a steady increase (da Silva et al 2003, Weiss et al 2005, Fevang et al 2007). Definitive indications for THR are not well established but often include, in addition to demonstration of typical degenerative changes in hip radiographs, continuous hip pain, loss of mobility, and considerable disability, all refractory to conservative treatment including adequate pain medication, physiotherapy and weight reduction (Zhang et al 2005, 2008, Malmivaara et al 2007).

5.1.3. Incidence of THR operations

The number of THR operations performed annually has been increasing steadily and quite rapidly during the past two decades in all western countries (Havelin et al 2000, Lucht 2000, Puolakka et al 2001, Ostendorf et al 2002, Wells et al 2002, Dixon et al 2004, Kurtz et al 2005, Pedersen et al 2005). In OECD countries, for example, a 25% increase in the incidence of THR operations occurred between 2000 and 2009 (OECD Health data 2011). Similarly in Finland, the number of THR operations performed annually increased from about 5000 in 1996 to over 8000 in 2006 (National agency for Medicines 2009). Since then, operation numbers in Finland have plateaued and even slightly declined, with 7400 THR operations performed in 2010 (Perälä 2010). Especially noteworthy is the dramatic increase in the THR operation nubers done on relatively young patients; In the Finnish population, the incidence of THR opearions tripled (30 vs. 90 /100 000) between 1980 and 2007 in patients aged 50 to 59, likely due to broadening indications for surgery (Skyttä et al 2011).

Currently, the overall incidence of THR operations in western countries exceeds 150/100 000, ranging from 93 in Spain to 296 in Germany (OECD Health data 2011). Based on these figures, estimates are that the impressive number of 1 million THR operations are currently performed globally each year. The number of operations is

expected to continue to increase, partially due to population aging and a related increased prevalence of osteoarthritis, and also due to widening THR surgery indications (Birrell et al 1999). According to an estimate based on the US population, the demand for THR operations will increase as much as 174%, reaching almost 600 000 THR operations per year in the US alone, by 2030 (Kurtz et al 2007).

5.1.4. Outcomes of THR operation

Since its introduction, THR surgery has become hugely successful, and it can well be said that THR has revolutionized treatment of end-stage, disabling arthritis. The Finnish Medical Society Duodecim's journal *Duodecim* recently highlighted artificial joints as one of the "twelve wondrous achievements of modern medicine" comparable to antibiotics, organ transplants, and x-rays (Ylikorkala el al 2011). Outcome studies over the years have consistently shown that THR operations are a reliable and effective means to manage the pain and disability of end-stage arthritis patients (Fitzpatrick et al 1998, Ethgen et al 2004). Typically, a considerable decrease in pain and improvement in ability to function occurs post-operatively, as well as recovery of health-related quality of life to the level of control populations (Wiklund and Romanus 1991, O'Boyle et al 1992, Fitzpatrick et al 1998, Lucht 2000, Söderman et al 2001, Ethgen et al 2004, Malchau et al 2005, Rolfson et al 2011). The majority of patients are satisfied with their outcome and return to their previous work (Nunley et al 2011). Thus, it is not surprising that THR is also among the most cost-efficient surgical interventions of modern medicine, despite the operation's relatively high cost (Quintana et al 2006, Räsänen et al 2007).

5.1.5. Revision THR operations

Although THR is accepted as a reliable and very cost-effective means to treat end-stage arthritis, and its functional outcomes are good, the operation is not without complications. Although there exists considerable variation between THR-survival rates, in 10 to 20 years after the primary operation, typically between 5 and 20% of the inserted THRs require a revision (Kavanagh et al 1994, Furnes et al 2001, Puolakka 2001, Berry et al 2002, Older 2002, Malchau et al 2005, Mäkelä et al 2008a, 2008b, Corbett et al 2010, Hailer et al 2010,). Especially in younger, more active patients, long-term survival of hip replacements is limited, and risk for long-term implant loosening considerable (Callaghan et al 1998, Lucht 2000, Older 2002, Corbett et al 2010). These revision THR operations are often technically demanding and expensive, and show clinical outcomes poorer than for the primary THRs.

Despite new and more durable hip replacement materials and advancements in surgical technique, the demand for revision THR operations has shown a steady increase during the last two deacades (Dixon et al 2004, Pedersen et al 2005, Marshall et al 2008, National agency for Medicines 2009). For example, in the US between 1990 and 2002, the incidence of revision THRs increased from 9.5 to 15.2/100 000. Likewise in England between 1991 and 2001, revision THR numbers doubled (Dixon et al 2004, Kurtz et al 2005). Finland currently has about 1000 THR revision operations annually, and although the revision numbers have remained about the same for the last decade, they are, based on similar trends in other western countries, expected to double in the next 10 to 20 years (National agency for Medicines 2009). This increase in revision THR operation numbers likely reflects the increasing numbers of primary operations performed and also the fact that the primary operations have been increasingly performed on younger and more active patients.

Most revision operations are performed because of aseptic (40-60%) or septic (10%) loosening of hip replacement components, although recurrent dislocation of the hip replacement (15%) is also one of the main reasons for revision operations (Lucht 2000, Puolakka 2001, Ulrich et al 2007, Bozic et al 2009, National agency for Medicines 2009).

5.2. Septic loosening of THRs

5.2.1. Definition, epidemiology, diagnosis

Septic loosening of THR refers to osteolysis and loosening primarily caused by bacterial infection of hip replacement components (Zimmerli et al 2004). Implant-related infection is a severe complication of THR surgery, which often necessitates removal of an infected implant and long-term antibiotic treatment before a new implant can be inserted, resulting in considerable expense and morbidity (Zimmerli et al 2004). Accordingly, extensive measures are undertaken to prevent this devastating complication. Currently, the risk for developing infection after THR is less than 1% (Phillips et al 2006, Pulido et al 2008, Huotari et al 2010, Jämsen et al 2010).

Recognized risk factors for implant-related infections include both surgery-related factors like duration of the operation and patient-related factors like smoking, diabetes with high blood glucose, and obesity (Jämsen et al 2010). About half the infections are caused by coagulase-negative staphylococci such as *Staphylococcus epidermidis*, and by *Staphylococcus aureus*. Other less commonly encountered pathogens include streptococcus and, in a minority of cases, gram-negative bacteria such as enterococci, *Escherichia coli, Pseudomonas aeruginosa*, or even more rarely anaerobic bacteria (Bernard at al 2004, Sia et al 2005, Phillips et al 2006, Esposito and Leone 2008, Pulido et al 2008). Based on the clinical picture and time of apparent onset, post-operative joint replacement infections are classically divided into three groups: early (occurring <3 months post-operatively), delayed (3-24 months after surgery), and late (>24 months afterwards) (Coventry 1975, Zimmerli et al 2004).

Early implant infections are caused by virulent bacteria like *Staphylococcus aureus* that gain access to the implant during, or immediately after, the primary surgery. Bacteria of high virulence can reach the joint replacement via hematogenous spread from body surfaces even years after the primary surgery and cause acute joint-replacement infection (late, acute infections). In both instances, the patient typically presents with both local and systemic signs and symptoms of an acute infection including fever, hip pain, and apparent surgical site infection with local cellulitis, and occasionally even formation of a sinus track with a purulent discharge (Zimmerli et al 2004, Sia et al 2005, Trampuz and Widmer 2006).

Diagnosis of this type of acute, early or late, joint replacement infection rarely offers particular difficulties, whereas diagnosis of delayed, low-grade implant infections may present considerable challenges. Such infections are typically caused by slow-growing bacteria of relatively low virulence but having the ability to effectively form implant-related biofilms (for example coagulase-negative staphylococci) (Zimmerli et al 2004, Sia et al 2005, Trampuz and Widmer 2006). The clinical presentation of these infections is typically mild, with obvious symptoms and signs of infection often absent. The patient typically presents with chronic hip pain and possibly with loosening of THR components, as evident in radiography.

As the clinical presentation of low-grade joint replacement infection is not self-evident and also overlaps with aseptic osteolysis, several attempts have been made to develop reliable diagnostic methods for such infections. Routine laboratory blood tests

including white blood cell count, C-reactive protein, and erythrocyte sedimentation rate, although often elevated during acute joint replacement infection, are rarely useful in the diagnosis of low-grade infection due to their low sensitivity (Bernard et al 2004, Zimmerli et al 2004, Sia et al 2005, Esposito and Leone 2008, Berbari et al 2010). Similarly, plain radiographs have low sensitivity and specificity in these cases.

Preoperative joint fluid aspirates, sometimes taken repeatedly, are a commonly used method in evaluation of suspected low-grade joint-replacement infection. Gram's staining and bacterial culture of aspirated synovial fluid have high specificity but low sensitivity (Bernard et al 2004, Zimmerli et al 2004, Sia et al 2005, Esposito and Leone 2008). In contrast, increased synovial fluid leukocyte count and an increased proportion of neutrophils in the differential count have high sensitivity and specificity for implant-related infection and have thus shown great promise as a practical diagnostic tool for low-grade hip- and knee-replacement infection (Spangehl et al 1999, Trampuz et al 2004, Ghanem et al 2008, Schinsky et al 2008, Cipriano et al 2012).

The gold standard for diagnosis of implant-related infection, peri-implant-tissue bacterial culture, is subject to false-positive results due to skin contaminants. The risk for these false-positives is reduced and an almost 100% specificity achieved if the same bacteria can be cultured from more than two peri-implant tissue samples (Zimmerli et al 2004, Sia et al 2005, Bauer et al 2006, Esposito and Leone 2008). The major weakness of peri-implant bacterial cultures is, however, that the method is also subject to false-negative results; bacteria causing low-grade implant infections typically grow slowly and poorly, at least in culture, and they also attach firmly to the implant surface, so that at any time point only small number of bacteria are released into tissues. Accordingly, peri-implant bacterial cultures have relatively low sensitivities ranging from 60 to 94% for diagnosis of implant-related infection (Zimmerli et al 2004, Sia et al 2005, Bauer et al 2006, Esposito and Leone 2008). Sensitivity of these intra-operative bacterial cultures is, furthermore, strongly affected by prior antibiotic treatment.

In addition to intra-operative bacterial cultures, histopathological examination of peri-implant tissue samples is a common method for diagnosis of joint-replacement infection. Observation of acute inflammation in intra-operative frozen sections (defined as 1-10 neutrophils per high-power field) has a specificity of more than 90% for implant infection but a very variable and generally low sensitivity (Lonner et al 1996, Pace et al 1997, Della Valle et al 1999, Spangehl et al 1999, Banit et al 2002, Francés Borrego et al 2006, Nuñez et al 2007, Bori G et al 2007, Kanner et al 2008). Histological examination is also challenging, because the amounts of inflammatory infiltrates can vary considerably between samples and between specific tissue areas examined and are also subject to interobserver variability.

Several scintigraphy techniques using various radiolabeled markers of infection have also been developed for diagnosis of joint replacement infection, but at least thus far these methods have shown no particular advantage over joint fluid aspirates or peri-implant bacterial cultures (Zimmerli et al 2004, Sia et al 2005, Bauer et al 2006).

No currently available method for detection of implant-related infection offers completely satisfactory combination of high sensitivity, specificity, and practicality. Thus in clinical practice, diagnostic methods are combined to support clinical judgement and to confirm or rule out low-grade joint replacement infection. For example, the working group of the Musculoskeletal Infection Society has recently proposed that joint replacement infection exists when:

1) A sinus tract is communicating with the implant; or
2) A pathogen is isolated by culture from at least two separate tissue or fluid samples from the affected prosthetic joint; or
3) When four of the following six criteria exist:
    a) Elevated serum erythrocyte sedimentation rate and serum C-reactive protein concentration;
    b) Elevated synovial leukocyte count;
    c) Elevated synovial neutrophil percentage;
    d) Purulence in the affected joint;
    e) Isolation of a microorganism in one culture of periprosthetic tissue or fluid;
    f) More than five neutrophils per high-power field in five high-power fields in histological analysis of periprosthetic tissue at ×400 magnification;

Implant infection may be present even with fewer than four of these criteria (Parvizi et al 2011).

5.2.2. Bacterial biofilm

Diagnostic and therapeutic difficulties typical for implant-related infection result mainly from formation of bacterial biofilm on the surface of implanted material (Costerton et al 1999, Trampuz et al 2003, Zimmerli et al 2004). Biofilms are complex multicellular and self-organizing structures resembling in many instances eukaryote multicellular organisms; bacteria in biofilms share metabolic pathways, communicate, and collectively regulate a multitude of physiological activities (Dunne 2002, Donlan 2002). Some biofilms even have fluid channels resembling primitive circulation and allow fluid and nutrition flow through the structure. Biofilm formation is a common property of prokaryote life found widely in different ecosystems, and is also typically utilized by common human pathogenic bacteria such as coagulase-negative staphylococci and *Staphylococcus aureus* (Donlan and Costerton 2002, Hall-Stoodley et al 2004). Likewise, these bacteria are able to attach to a wide variety of surfaces, including all currently used implant materials (Zimmerli and Sendi 2011).

Bacteria in biofilm attach tightly to the underlying material or more specifically to its protein coating; they multiply and produce a polymeric extracellular matrix composed primarily of anionic polysaccharides that effectively protect them from environmental challenges and also the from host immune system including phagocytes, the complement system, and antibodies (Costerton et al 1999, Donlan and Costerton 2002, Zimmerli and Sendi 2011). This crippling of the host immune response is exemplified by the fact that less-virulent bacteria are able to cause considerable implant-related infection and, on the other hand, that the minimum number of microbes that can cause infection is dramatically reduced (Zimmerli and Sendi 2011).

In addition to the host immune system, biofilms are resistant to antibiotics in part due to reduced penetration of antibiotics into biofilm, extreme microenvironmental conditions residing inside the biofilm (e.g. low pH), and also in part due to the presence of very slowly growing bacterial populations inside the structure (Donlan and Costerton 2002, Dunne WM 2002, Hall-Stoodley et al 2004). Thus, although some parts of the biofilm may be lost due to the host immune response or antibiotic treatment, others prevail and cause repeated outbursts of infection necessitating implant removal to control the infection. Likewise, bacteria that grow on the surface of the implant inside the biofilm are not necessarily detected by conventional bacterial culture samples taken from the peri-implant tissues (Trampuz et al 2007).

The natural course and prognosis of this kind of low-grade, biofilm-hidden implant infection is variable and currently poorly understood (Donlan and Costerton 2002). It has been postulated that such an infection may remain quiescent and go mostly undetected by the host immune system for years while low levels of bacteria released from the biofilm are causing low grade peri-implant inflammation and eventual osteolysis. As the biofilm-hiden bacteria are easily missed by conventional bacterial culture methods, what has been speculated is that a quiescent joint replacement infection may produce a clinical picture overlapping or even indistinguishable from aseptic loosening (Nelson et al 2005).

5.3. Aseptic loosening of THRs

5.3.1. Definition

Aseptic loosening of THRs refers to a sequence of events in which hip replacement that has remained stable and osseointegrated for an extended period of time, possibly even for a decade or two, becomes loosened, as the bone surrounding one or more of the THR components is resorbed. This resorbed bone is replaced with loose connective tissue heavily infiltrated with macrophages and foreign-body giant cells. This tissue is known as synovial membrane-like tissue or interface tissue.

A patient suffering from aseptic loosening typically presents with hip pain and, in the most advanced cases, with mechanical instability and shortening of the corresponding lower extremity. Development of osteolytic lesions might, however, also be asymptomatic and does not necessarily lead to joint-replacement loosening. In these cases, osteolytic lesions that might be substantial become evident only in follow-up radiographs. This is typically the case with well-osseointegrated uncemented joint replacements, which may develop considerable osteolytic lesions while retaining their relatively firm fixation to remaining bone.

Hip radiography shows osteolytic radiolucent lesions surrounding THR, and in the case of loosening, may reveal migration of the implant components. Treatment of choice is a revision operation in which loosened THR components and accompanying interface tissue are removed and replaced with new components. Bone defects left by the osteolytic lesions can make fixation of new joint replacement components a technical challenge.

Osteolysis and aseptic loosening are the primary long-term complications of THR and the leading cause of revision operations, accounting for about two-thirds of THR revisions (Ulrich et al 2007, Bozic et al 2009, National Agency for Medicines 2009). Especially in younger patient groups, long-term THR survival is limited by aseptic loosening (Callaghan et al 1998, Older 2002, Corbett et al 2010).

5.3.2. Mechanisms of aseptic loosening

According to the traditional definition, loosening of the THR is termed aseptic when clinical signs or symptoms of an overt infection are absent, and the diagnostic criteria for septic loosening are unfulfilled, essentially meaning that no involvement of bacteria can be demonstrated in implant loosening or osteolysis (Holt et al 2007, Purdue et al 2007, Goodman et al 2009, Gallo et al 2012). Although it is often said that Charnley himself speculated that aseptic implant failures might be actually caused by subclinical infection, since his era, involvement of bacteria in the process of aseptic loosening has been excluded by definition, and other mechanisms of this phenomenon have been vigorously sought (Learmonth et al 2007).

Suggested mechanisms of aseptic loosening include high oscillating joint fluid pressure, stress shielding, and micromotion between the implant and surrounding bone

(Sundfeldt et al 2006). The best established of these, however, is the “particle disease” theory (Willert and Semlitsch 1977, Harris 1995, 2001, Ingham and Fisher 2005, Konttinen et al 2005, Purdue et al 2006, 2007, Holt et al 2007, Goodman et al 2009, Gallo et al 2012). Due to unavoidable abrasion between the hip replacement bearing surfaces and implant and bone, hip replacement components slowly wear and erode over time, and high amounts of foreign-material wear particles are released into the pseudosynovial fluid, surrounding tissues, or both. The exact size, shape, and chemical nature of these wear particles varies depending on their origin. Most commonly, however, they are UHMWPE particles released from the bearing surface as the femoral metal head slowly abrades the UHMWPE liner. Conventional, well-functioning UHMWPE cup liners show a mean linear wear rate of about 0.1 mm/year, leading to generation of a vast amount of UHMWPE particles (Ilchmann et al 1998, Sochart 1999, Dowd et al 2000, Dumbleton et al 2002). Metal-on-metal implants display considerably different wear behavior, with diminished volumetric wear but again with release of vast amounts of nano-sized metal particles and metal into the interface tissue (Doorn et al 1998, Jacobs and Hallab 2006). Additional wear-debris sources include fragmentation and delamination of PMMA surrounding the implant, as well as metal or ceramic particles released from alternative bearing surfaces or from implants surface modifications due to fretting wear (Harris 2001, Holt et al 2007, Purdue et al 2007, Goodman et al 2009).

Particles released into the pseudosynovial fluid are distributed into the surrounding tissues by pressure waves generated in the fluid while the artificial joint is being used, and typically the interface tissue is highly loaded with biomaterial wear-particles of various types (Willert and Semlitsch 1977, Schmalzried et al 1992, Margevicius et al 1994, Maloney et al 1995, Hirakawa et al 1996). Retrieval analyses have shown that UHMWPE wear particles show considerable size and morphological variability, most commonly, however, being spheroids with mean diameter of about 0.5 to 0.7 µm and with the vast majority of particles being between 0.1 and 1 µm (Shanbhag et al 1994a, Campbell et al 1995, Tipper et al 2000, Howling et al 2001, Koseki et al 2005).

It is generally accepted that this high-wear particle load causes a foreign-body reaction, subsequent chronic low-grade inflammation, and ultimately osteolysis (Willert and Semlitsch 1977, Harris 1995, 2001, Ingham and Fisher 2005, Konttinen et al 2005, Purdue et al 2006, Holt et al 2007, Purdue et al 2007, Goodman et al 2009, Gallo et al 2012). Massive macrophage infiltrates and formation of foreign-body giant cells as well as ostoclasts at the bone-soft tissue interface are hallmarks of the foreign body reaction, typically seen also in the interface tissue surrounding the loosening implant (Figure 2). The basic notion of the particle disease hypothesis is that the huge amount of wear particles generated are, in the forming interface tissue, phagocytosed by macrophages which respond to this foreign material by production of inflammatory cytokines, chemokines, and growth factors that lead to recruitment of further macrophages into the interface tissue via endothelial activation and chemotaxis. Further, macrophage-derived inflammatory mediators lead to local imbalance of the receptor activator of the nuclear factor-kB ligand (RANKL) and osteoprotegerin (OPG) system. This increased RANKL/OPG ratio, along with the inflammatory cytokines produced, creates a microenvironment that favors osteoclastogenesis and osteolysis, finally leading to implant loosening (Figure 3). Additionally, chronic inflammation and macrophage activation in peri-implant tissues may lead to acidification of the interface tissue, to a pH sufficiently low to cause bone demineralization directly (Konttinen et al 2001).

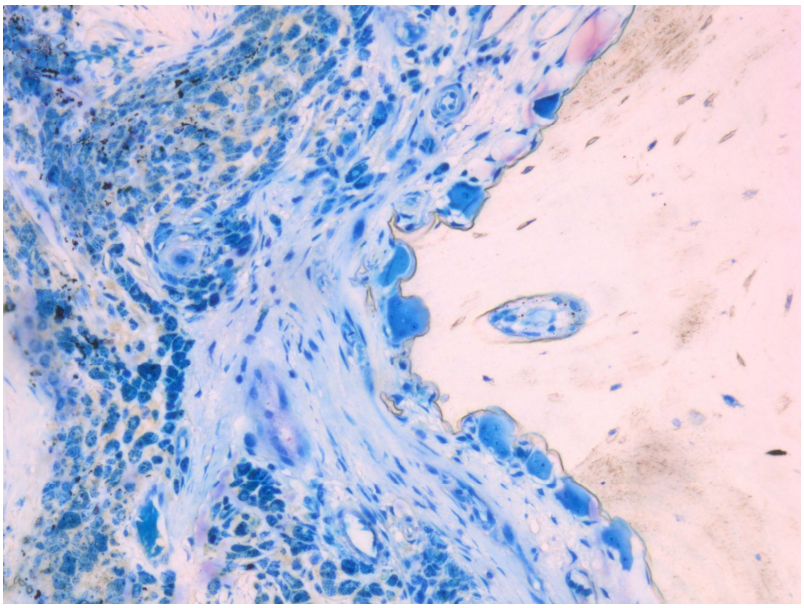

Figure 2. Osteolytic lesions in aseptic loosening are composed of hypertrophic interface tissue typically from the implant side covered by pseudosynovial membrane. The underlying well-vascularized loose connective tissue is heavily infiltrated by macrophages organized into sheet-like formations. High amounts of metal, UHMWPE, or other wear particles are phagocytosed by macrophages. Some of the macrophages have fused to form multinucleated foreign-body giant cells that often surround larger foreign bodies. Osteoclast formation and active bone resorption is observed at the soft tissue-bone interface

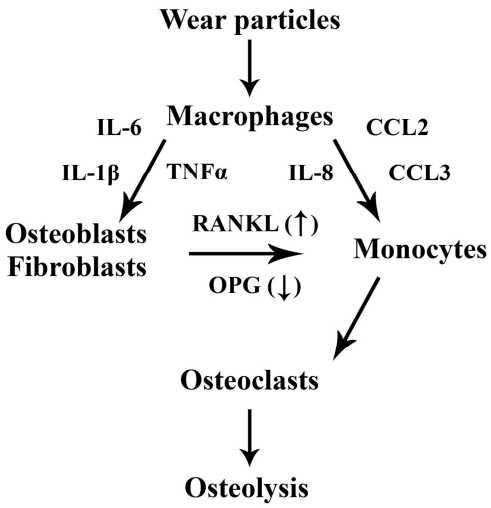

Figure 3. The "particle disease" theory in brief: Macrophages are activated by wear particles to produce various chemokines and inflammatory mediators. Chemokines recruit additional monocytes into interface tissue, and inflammatory cytokines lead both directly and indirectly to increased ostoclastogenesis, bone resorption, and implant loosening. The exact mechanisms by which wear particles are recognized by macrophages and cause macrophage activation are poorly understood. TNFα - tumor necrosis factor alpha; IL - interleukin; CCL - chemokine (C-C motif) ligand; RANKL - receptor activator of nuclear factor kappa B ligand; OPG - osteoprotegerin.

Several lines of evidence support this possible sequence of events. First, risk for developing aseptic osteolysis and implant loosening is directly proportional to the annual UHMWPE linear wear rates and to the corresponding dose of wear particles released into the peri-implant tissues (Sochart 1999, Han et al 1999, Dowd et al 2000, Dumbleton 2002, Wilkinson et al 2005, Emms et al 2010).

Second, interface tissue is characterized by massive macrophage infiltrates and foreign body giant cells that typically contain large amounts of phagocytosed particulate material (Willert and Semlitsch 1977, Goldring et al 1983, Goodman et al 1989, Santavirta et al 1990, Jiranek et al 1993, Kim et al 1993, Boynton et al 1995, Goodman et al 1997, Goodman et al 1998).

Third, numerous studies have characterized the inflammatory nature of interface tissue and documented the increased production of a large array of primarily macrophage-derived pro-inflammatory cytokines, chemokines, and growth factors including tumor necrosis factor alpha (TNFα), interleukin (IL)-1β, IL-6, IL-8, macrophage colony-stimulating factor (M-CSF), granulocyte-macrophage colony-stimulating factor (GM-CSF), vascular endothelial growth factor (VEGF), chemokine (C-C motif) ligand (CCL) 2, CCL3, and prostaglandin E2 (PGE2) in the interface tissue (Kim et al 1993, Sabokbar et al 1995, Chiba et al 1994, Xu et al 1996, Ishiguro et al 1997, Xu et al 1997, Goodman et al 1998, 2010, Takei et al 2000, Lassus et al 2000, Stea et al 2000, Konttinen et al 2002, Spanogle et al 2006, Wang et al 2010). Interface tissue is furthermore characterized by increased production of several matrix metalloproteinases (MMPs) and cathepsin K that possibly participate in local tissue destruction or remodeling (Takagi et al 1994a, 1994b, 1995, 1998, Konttinen et al 2001). Increased production of RANKL and decreased or unaltered production of OPG is also detectable in interface tissue, and this change in RANKL/OPR ratio is likely responsible for the formaton of osteoclasts and foreign body giant cells (Mandelin et al 2003, Clohisy et al 2003, Horiki et al 2004, Crotti et al 2004, Holding et al 2006, Wang et al 2010). In agreement with these observations, pseudosynovial fluid from aseptically loosened implants induces formation of osteoclasts in vitro, an effect likely due to soluble RANKL contained in the fluid, because added OPG reduces osteoclast formation (Kim et al 2001, Mandelin et al 2005b).

Fourth, in various animal model systems, various foreign material particles, including PMMA, UHMWPE, and titanium, cause osteolysis, accompanied by upregulation of inflammatory cytokines and an increased RANKL/OPG ratio (Goodman et al 1990, Spector et al 1990, Gelb et al 1994, Merkel et al 1999, Schwarz et al 2000a, Wooley et al 2002, Warme et al 2004, Masui et al 2005, Ren et al 2011). This particle-induced osteolysis can be reduced by inhibiting TNFα signaling either by use of a TNFα-neutralizing antibody or by deleting the TNF receptor. These observations highlight the role of TNFα as a key mediator in osteolysis (Merkel et al 1999, Childs et al 2001a, 2001b, Schwarz et al 2000b). Likewise, in murine model systems, blocking of RANKL signaling either by a RANKL antibody or by OPG diminishes particle-induced osteolysis, which is also diminished in mice genetically lacking receptor RANK (Childs et al 2002, Ulrich-Vinther et al 2002, Yang et al 2002a, Goater et al 2002).

Finally, in vitro studies have shown that monocyte-macrophages challenged with various types of wear particles are activated to produce a wide variety of pro-inflammatory cytokines (TNFα, IL-1β, IL-6, PGE2), chemokines (IL-8, CCL2, CCL3), growth factors (VEGF, M-CSF, GM-CSF), and MMPs (MMP1, MMP2, MMP9) (Herman et al 1989, Murray et al 1990, Glant et al 1993, Shanbhag et al 1995, Maloney et al 1996, Blaine et al 1996, Nakashima et al 1998, 1999a, 1999b, Matthews et al 2000a, 2000b, Hatton et al 2003). They also show that supernatants from these cultures can induce

osteolysis in both in vitro and in vivo assays. The magnitude of the inflammatory and osteolytic effect depends on amount and volume of particles as well as on their shape, size, and exact biomaterial composition (Gelb et al 1994, Shanbhag et al 1994b, Haynes et al 1998, Green et al 1998, 2000, Yang et al 2002b, Sethi et al 2003, Ingram et al 2004).

Further in vitro studies using other relevant cell types of interface tissue, namely fibroblasts and osteoblasts, have demonstrated that to some extent, wear particles directly and especially macrophage-derived pro-inflammatory cytokines up-regulate RANKL production from interface tissue fibroblasts and osteoblasts. This effect provides a direct link between wear-particle-induced macrophage activation and increased production of RANKL and subsequent osteoclastogenesis (Quinn et al 2000, Mandelin et al 2005a, Wei et al 2005, Sabokbar et al 2005, Koreny et al 2006). Wear particles also directly suppress osteoblast formation and function and to some extent also directly up-regulate RANKL production from osteoblasts, thus directly inhibiting bone formation and promoting its resorption (Dean et al 1999a, 1999b, Vermes et al 2000, 2001a, 2001b, Fritz et al 2002, Wang et al 2002, Pioletti et al 2002, Pioletti and Kottelat 2004). Likewise, wear particles are also able to directly activate interface tissue fibroblasts to produce inflammatory and chemotactic mediators as well as MMPs (Yao et al 1995, Manlapaz et al 1996, Yaszay et al 2001).

Further insight into the mechanisms of aseptic loosening has come through genetic association studies demonstrating that risk for developing aseptic osteolysis is associated with certain polymorphisms in cytokines (TNFα, IL-6 transforming growth factor beta, TGFβ), matrix metalloproteinases (MMP1, MMP2), and OPG, thus further highlighting the underlying inflammatory mechanisms of aseptic osteolysis and implant loosening (Del Buono et al 2012).

5.3.3. Suspected role bacterial products in aseptic loosening

In recent years, the strict distinction between septic and aseptic hip replacement loosening has again been questioned, as bacterial structural products or even biofilms can be detected in at least some of the seemingly aseptic interface tissues and explanted implants, by means of special sampling methods such as the polymerase chain reaction, sonication of explanted implants, or prolonged bacterial cultures (Tunney et al 1998 and 1999, Nguyen et al 2002, Clarke et al 2004, Nelson et al 2005, Esteban et al 2008, 2012, Kobayashi et al 2008, Schäfer et al 2008, Sierra et al 2011, Portillo et al 2012). The most convincing support for this till-now unrecognized role of subclinical bacterial infection in pathogenesis of aseptic loosening comes from observations that occurrence of “aseptic” long-term hip-replacement loosening is reduced by use of intra-operative antibiotic prophylaxis and antibiotic-loaded bone cement (Espehaug et al 1997, Engesaeter et al 2003).

Subsequent in vitro and in vivo studies have shown that the various wear particles are, in fact, relatively inert and cause only limited macrophage activation and osteolysis and that their inflammatory and osteolytic properties largely depend on bacterial structural components adhering to their surfaces (Ragab et al 1999, Daniels et al 2000, Bi et al 2001a, 2001b, 2002, Brooks et al 2002, Cho et al 2002). Due to its easy-to-use and sensitive detection method (Limulus assay), bacterial LPS has, in most of these studies, served as a model for any bacterial-derived molecule that might adhere to and concentrate on wear-particle surfaces (Greenfield et al 2005). In an in vivo setting, in addition to subclinical biofilms, such bacterial structural components might find their way into the interface tissue via hematogenous spread, being originally released into circulation from minor infections in body surfaces such as periodontal tissue, skin, and urinary- and

gastrointestinal tracts. In interface tissue these bacterial products then adhere to, and concentrate on the hydrophobic surfaces of wear particles (Greenfield et al 2005). This hypothesis is supported by observations that LPS is periodically found in the circulation of healthy individuals and that at least in a mouse model of particle induced-osteolysis, LPS accumulates on the wear particles of the interface tissue (Xing et al 2006, Tatro et al 2007). Additionally, orthopedic implants may already be contaminated with variable amounts of LPS during the manufacturing process, a contamination difficult to remove by standard sterilization procedures (Ragab et al 1999, Bonsignore et al 2012). In agreement with these observations, LPS is detectable in at least some seemingly aseptic interface tissues (Nalepka et al 2006). There thus seems to be a clear overlap between low-grade hip replacement infection and aseptic loosening.

5.4. Monocyte-macrophages

5.4.1. General features

Macrophages were identified in the second half of the 19th century by Russian scientist Elie Metchnikoff, who, in addition to identifying and naming these cells, characterized their key function, phagocytosis, and described its role in both normal tissue homeostasis and in immunity (Gordon 2008, Kaufmann 2008). Metchnikoff received the 1908 Nobel Prize in medicine for these discoveries, thus setting in motion vigorous research on innate immunity. Since then, it has become clear that macrophages are very versatile, multipurpose, and dynamic cells that play a key role in embryonic development, in normal tissue homeostasis, in orchestration of inflammation reaction, in activation of adaptive immunity, and in initiation as well as orchestration of tissue healing (Gordon 2007). Likewise, aberrant macrophage function has been implicated in the pathogenesis of several major non-infectious but inflammatory disease groups including atherosclerosis, arthritis, and malignancies as well as several major infectious diseases (Kinne et al 2007, Solinas et al 2009, Murray and Wynn 2011b, Shalhoub et al 2011). In the context of foreign-body reactions to implanted biomaterials, macrophages are considered the main mediators of biomaterial and implant rejection (Ingham and Fisher 2005, Anderson et al 2008). Discovery of pattern-recognition receptors (PRRs) during the past two decades has further highlighted the key role that macrophages play in both health and disease (Gordon 2007).

Macrophages are professional phagocyte cells of the innate immune system. They are produced in red bone marrow and released into the circulation as precursors called monocytes, which home to various tissues under steady-state conditions and differentiate into mature macrophages under the guidance of various signals in the local micro-environment (Geissmann 2010b, Galli et al 2011). Most tissues contain these highly specialized macrophage populations, termed resident macrophages, or M0 macrophages, that participate in normal tissue homeostasis, housekeeping, immunosurveillance, recognition of pathogen invasion, and sensing of sterile tissue damage (Murray and Wynn 2011b). Collectively, this widespread network of tissue-resident macrophages is known as the mononuclear phagocyte system (Ross and Pawlina 2011a).

During acute tissue inflammation following neutrophil invasion, monocytes adhering to and transmigrating through activated endothelium form the second wave of the innate immune response. Once in the inflamed tissues, monocytes mature into macrophages which then, due to host- and pathogen derived micro-environmental signals, become activated and subsequently vigorously engage their effector functions which include phagocytosis of cell debris and pathogens as well as secretion of inflammatory

mediators: such macrophages are known as inflammatory, classically activated, or M1 macrophages (Mosser and Edwards 2008). Once a tissue insult is cleared by the conjoint effort of neutrophils and macrophages, M1 macrophages switch their mode of function to support healing and regeneration: macrophages effective in these functions are known as regulatory, alternatively activated, or M2 macrophages (Mosser and Edwards 2008). If tissue insults cannot be cleared by the innate immunity, the inflammation persists, and the adaptive immunity is subsequently activated.

Central features of macrophage function include phagocytosis of cell debris and invading pathogens as well as subsequent killing of phagocytosed pathogens by effective production of an array of microbicidal mediators (Gordon 2007, Mosser and Edwards 2008, Murray and Wynn 2011b). Additionally, macrophages are effective secretory cells that participate in regulation of the inflammation reaction by carefully orchestrated secretion of both pro- and anti-inflammatory cytokines and chemokines. Subsequent tissue healing is, at least in part, attributable to various growth factors secreted by macrophages. Additional macrophage characteristics are considerable functional plasticity and phenotypical adaptability to various signals derived from the local micro-environment (Gordon 2007, Mosser and Edwards 2008, Galli et al 2011, Murray and Wynn 2011b).

Rapid migration to the local lymph nodes and effective antigen presentation to the T lymphocytes in the context of sufficient expression of co-stimulatory molecules, and the subsequent activation of the adaptive immune response, are attributes that currently most clearly distinguish dendritic cells from macrophages (Geissmann et al 2010b). Nevertheless, macrophages also express major histocompatibility complex (MHC) I and II molecules and are able to present antigens to activated and immunocompetent T lymphocytes that reciprocally coordinate macrophage function by secreting interferon gamma (IFNγ) or IL-4 (Geissmann et al 2010a). Macrophages are thus important effector cells in the cell-mediated arm of adaptive immunity. Equally, high expression of Fc-receptors allows macrophages to effectively recognize antibody-opsonized pathogens and toxins; thus they function as effector cells also for the humoral arm of the adaptive immune system.

5.4.2. Myeloid development and blood monocytes

Cells of monocyte-macrophage lineage originate from a self-renewing pool of multipotent hematopoietic stem cells residing in the red bone marrow (Geissmann 2010b). Hematopoietic stem cells give rise to common lymphoid and myeloid progenitors which then differentiate into mature lymphoid and myeloid lineage cells via successive commitment steps with increasingly restricted differentiation potential. Intermediates in this monocyte-macrophage hematopoietic development lineage include common myeloid progenitors, granulocyte-macrophage progenitors, monoblasts, promonocytes, and finally monocytes that are released into the circulation (Auffray et al 2009). This process of monocytopoiesis takes about 2 days and is dependent, at least in mice, on expression of the colony-stimulating factor 1 receptor, ligands of which are M-CSF and IL-32, while the role of other cytokines like GM-CSF is considered to be primarily modulatory (Auffray et al 2009). Several transcription factors have been identified in monocytopoiesis, the most important of which is PU.1 (Lawrence and Natoli 2011). Continuous high expression of PU.1 is required throughout monocytopoiesis, and it apparently defines monocyte-macrophage cell identity (or the macrophage-specific global genomic landscape) by regulating available enhancer-binding sites on deoxyribonucleic acid (DNA). All other transcription factors regulating subsequent monocyte-macrophage differentiation and activation steps function in the general context defined by the PU.1.

Monocytes account for 1 to 10% of circulating leukocytes (Auffray et al 2009). They remain in circulation for various periods, typically about 3 days (Ross and Pawlina 2011a). In blood-smear preparations, monocytes are readily distinguishable from other mononuclear leukocytes by their relatively large size, lack of apparent granules, kidney-shaped or indented nuclei, and their large cytoplasm-to-nucleus ratio (Ross and Pawlina 2011a). For three decades, what has, however, been recognized is that monocytes are a heterogenous and functionally diverse population of cells (Gordon and Taylor 2005, Auffray et al 2009, Geissmann 2010b). About 5% of circulating monocytes represent tissue dendritic cell precursors, and the remaining 95% are "actual" monocytes that can be further divided into two broad categories based on morphological and functional features and specific cell-surface markers. The larger fraction, comprising about 90% of actual monocytes, is made up of large, "classical", $CD14^+CD16^-$ monocytes showing high phagocytic activity, a suppressed ability to produce pro-inflammatory cytokines, and a correspondingly higher tendency to produce anti-inflammatory cytokines in response to PRR stimulation. The remaining monocyte fraction is formed by small "non-classical" $CD14^+CD16^+$ monocytes with a high inherent capacity to produce inflammatory cytokines and an enhanced ability for antigen presentation (Ziegler-Heitbrock 2007).

Despite the fact that the existence of these monocyte populations has been known for some time, they are still relatively poorly characterized, in particular, with their roles in health and disease poorly understood. According to an established model, $CD14^+CD16^-$ monocytes home to tissues under steady state conditions and preferably differentiate into M0 or M2 macrophages, thus representing the precursors of resident macrophages, whereas $CD14^+CD16^+$ monocytes home primary to inflamed tissues, where they differentiate preferably into M1 macrophages and inflammatory dendritic cells (Gordon and Taylor 2005, Auffray 2009, Geissmann 2010b). This model, although likely oversimplified, is supported by clinical observations that the circulating numbers of $CD14^+CD16^+$ monocytes increase during infectious and inflammatory disorders, and also by mouse studies in which corresponding murine inflammatory monocyte populations (often designated as $Ly6^+/Grl^+$ monocytes) are recruited to tissue in early stages of inflammation (Mizuno et al 2005, Arnold et al 2007, Auffray et al 2007, Nahrendorf et al 2007, Ziegler-Heitbrock 2007). As inflammation evolves towards healing, $Ly6^-/Grl^-$ monocytes, resembling human $CD14^+CD16^-$ monocytes and with a more M2-like phenotype, are recruited into regenerating tissue; once there, they promote tissue healing and regeneration (Arnold et al 2007, Auffray et al 2007, Nahrendorf et al 2007).

Tissue-resident macrophage populations may originate primary from blood $CD14^+CD16^-$ monocytes. This matter is, however, complicated by discoveries that at least some (Langerhans cells of the epidermis and microglia) tissue-resident macrophage or dendritic cell populations are self-renewing; under steady state conditions they repopulate themselves from the circulating monocyte pool, but only slowly, if at all (Merad et al 2002, Ajami et al 2007). Thus, the complex dynamics of the monocyte-macrophage system, especially the origins and maintenance of tissue-resident macrophage populations, is still poorly understood. What is, however, generally agreed upon is that considerable infiltration of blood monocytes into peripheral tissues occurs during tissue inflammation (Gordon and Taylor 2005, Auffray et al 2009, Geissmann 2010b).

5.4.3. Tissue-resident macrophages

Macrophages are present in most tissues under steady state conditions as highly specialized and tissue-specific subpopulations, as exemplified by liver Kupffler cells, microglia of the central nervous system, histiocytes of the connective tissue, pulmonary

alveolar macrophages, and type B lining cells of the synovial tissue (Gordon and Taylor 2005, Murray and Wynn 2011b). Resident macrophage populations can comprise as much as 10 to 20% of the total number of tissue cells. These tissue-specific resident macrophage populations participate both in general tissue housekeeping and in regulation and maintenance of the local tissue specific-micro-environment (Pollard 2009, Galli et al 2011, Murray and Wynn 2011b).

One of the most important functions of resident macrophages is clearance of the impressive number of apoptotic cell bodies (estimated to be more than $>10^9$ cells per day) created daily throughout the system (Elliott and Ravichandran 2010). This rapid migration to and recognition of apoptotic bodies, and the subsequent phagocytosis and digestion along the endolysosomic pathway is mediated by release of chemotactic mediators from apoptotic cells and a specific set of cell-surface receptors, e.g. Bai1, Tim-4 and Stabilin-2 expressed on macrophages (Elliott and Ravichandran 2010). Signaling via these receptors leads to production of anti-inflammatory cytokines like IL-10 and TGFβ. This rapid clearance of apoptotic cells without inflammation is imperative for maintenance of immunological tolerance, as highlighted by the fact that inefficient removal of apoptotic bodies and subsequent secondary necrosis of these bodies has been linked to several different autoimmune diseases (Elliott and Ravichandran 2010). Likewise, tissue-resident macrophages participate in ECM turnover by secreting proteianases and in regulation of local cell growth and differentiation by secreting growth factors (Pollard 2009, Murray and Wynn 2011b, Galli et al 2011).

Tissue-resident macrophages also continuously survey their environment for foreign bodies, pathogens, and other danger signals by means of various sets of cell-membrane, endosomal, and cytoplasmic receptors, including at least three sets of PRRs (including, importantly, TLRs) and a group of scavenger receptors, Fc-receptors, and complement receptors. These macrophage populations thus also form the first line of defense against invading pathogens and are important in initiating an inflammation reaction if activated in sufficient amounts by danger-signal molecules in a process known as macrophage activation (Murray and Wynn 2011b).

5.4.4. Macrophage activation

Macrophage activation, also called innate macrophage activation to make it distinct from the full M1 macrophage phenotype, refers to a complex set of macrophage responses elicited by their recognition of invading microbes or other danger signals (Gordon 2003, Martinez et al 2008, Mosser and Edwards 2008). Currently, macrophage activation is best understood in the context of TLR signaling. In brief, TLRs recognize various microbial and also host-derived danger-signal molecules. Subsequent signaling through various TLRs leads to activation of inflammatory transcription factors including interferon regulatory factor (IRF) 5, mitogen-activated protein kinases (MAPK), activator protein 1 (AP-1), and nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB), and possibly IRF3 (only TLR3 and TLR4) which are collectively responsible for the macrophage activation phenotype.

Using genome-wide microarray transcription profiling and human macrophage cultures challenged by various types of bacteria (gram-positive, gram-negative, and mycobacterium) it has been possible comprehensively to characterize the shared transcriptome changes induced by these three types of bacteria (Boldrick et al 2002, Nau et al 2002, Jenner and Young 2005). These transcriptome changes, now collectively known as the macrophage activation program, consist of about 200 genes induced or suppressed in response to these bacterial challenges. Induced genes of the activation

program include pro-inflammatory cytokines and chemokines, membrane receptors, adhesion molecules, intracellular signaling components of PRRs, MMPs, and also reciprocal activation of anti-inflammatory factors that limit macrophage activation in the manner of negative feed-back loop (Boldrick et al 2002, Nau et al 2002, Jenner and Young 2005).

Importantly, the vast majority of transcriptional changes in a macrophage activation program induced in response to whole bacteria can be induced also by isolated bacterial structural components (LTA, LPS, lipoarabinomannan) which are ligands for different TLRs, thus indicating that the macrophage activation program actually is primarily formed by TLR-responsive elements, whereas the role of various other macrophage receptors seems, in comparison, rudimentary (Nau et al 2002).

5.4.5. Macrophage polarization

In addition to innate activation induced by TLR signaling, macrophages can assume distinct activation phenotypes as a response to the local cytokine microenvironment. Reflecting the concept and following the nomenclature of $CD4^+$ T helper lymphocyte polarization into Th1 and Th2 cells, a corresponding concept has been established of functional macrophage polarization into M1 and M2 macrophages, or "classically" and "alternatively" activated macrophages (Gordon 2003, Ma et al 2003, Mantovani et al 2004, Mosser and Edwards 2008, Galli et al 2011, Lawrence and Natoli 2011). According to this paradigm, M1 macrophages are related to and meditate Th1 polarized effector functions and M2 macrophages correspondingly to Th2 effector functions. Despite the apparent clarity of this definition, the nomenclature in the field is somewhat complicated and not well defined.

Classical macrophage activation referred originally to the macrophage phenotype that is induced by macrophage exposure to IFNγ and TNFα (Mosser and Edwards 2008). As the myeloid differentiation factor 88 (MyD88)-dependent TLR signaling pathway leads to auto- and paracrine production of TNFα, this original definition of M1-inducing signals has since been modified to include priming with IFNγ followed by activation to the full classical macrophage phenotype by TLR signaling (Mosser and Edwards 2008). The concept of the M1 macrophage has, however, continued to widen and is in the current literature used to describe macrophages primed with IFNγ (without TLR signaling) and also sometimes macrophages that have been differentiated with GM-CSF (Verreck et al 2004, 2006, Fleetwood et al 2007, Mosser and Edwards 2008, Lawrence and Natoli 2011). Furthermore, signaling via TLR3 and TLR4 induces production of type 1 interferons that have effects on macrophages somewhat similar as to those of IFNγ; thus, certain TLR ligands can induce the M1-like phenotype without any external source of INFγ (Mosser and Edwards 2008).

The original description of alternative macrophage activation referred to a macrophage phenotype induced by macrophage exposure to IL-4 (Stein et al 1992). This description of M2 macrophages was soon expanded to encompass macrophage exposure to closely related IL-13, and has since then been widened still more to include macrophages exposed to M-CSF that show some similarity to IL-4 exposed macrophages (Verreck et al 2004, 2006, Fleetwood et al 2007, Hamilton 2008, Lawrence and Natoli 2011).

It is generally agreed that M1 macrophages are proficient effector cells of Th1-related cell-mediated immunity and that they are effective producers of inflammatory mediators. M1 macrophages are also especially effective in killing phagocytosed pathogens that might otherwise be resistant to endolysosomal conditions and are effective

in antigen presentation (Martinez et al 2008). The role in immunity of M2 macrophages is less well defined and more multidimensional, ranging from parasite immunity to wound healing (Martinez et al 2009, Mosser and Edwards 2008). This functional and phenotypical definition of the M1 and M2 macrophage is, however, also problematic, because the phenotypes of human and mouse M1 and M2 macrophages have some major discrepancies (Martinez et al 2009, Murray and Wynn 2011a). For example, high nitric oxide production is typical for cultured mouse M1 macrophages, but not for cultured human M1 macrophages. Likewise, the enzyme arginase 1 is up-regulated in mouse M2 macrophages but apparently not in human macrophages.

5.4.6. Classical macrophage activation (M1 macrophages)

Despite the prevailing confusion over macrophage nomenclature, M1 macrophages refer to an inflammatory macrophage phenotype that is especially related to Th1 -response and immunity against intracellular pathogens. Priming with the Th1-derived pro-inflammatory cytokine INFγ is also fundamentally related to M1 phenotype.

INFγ is a pro-inflammatory cytokine secreted primary by innate or adaptive immune lymphocytes (Schroder et al 2004). During initial recognition of pathogens or danger signals via TLRs, antigen-presenting cells are induced to produce various pro-inflammatory cytokines such as IL-12 that recruit and activate natural killer lymphocytes. It is thought that these natural killer lymphocytes are responsible for initial production of INFγ and initial macrophage activation; additionally signaling through TLR3 and TLR4 leads to production of type 1 interferons, which then, acting in an auto and paracrine manner, can partially substitute for INFγ in inducing the M1 phenotype (van Boxel-Dezaire et al 2006, Mosser and Edwards 2008, Trinchieri 2010). Subsequently, IL-12 production polarizes the T helper-cell response towards the Th1-type, which then migrates to the tissue and produces large quantities of INFγ. This thus further guides macrophage activation (Schroder et al 2004, Mosser and Edwards 2008).

IFNγ binds to an IFNγ receptor, which then signals via the Janus-kinase signal transducer and activator of transcription (JAK-STAT) pathway, leading to activation of the transcription factors STAT1 and IRF5 (Schroder et al 2004, Lawrence and Natoli 2011). STAT1 is crucial for induction of the M1 phenotype and leads directly to transcription of the majority of M1-related genes, as does IRF5, the role of which is more modulatory.

Activation of these pathways has profound effects on the macrophage phenotype and leads to up-regulation of genes and macrophage functions important for cell-mediated immunity (Mosser and Edwards 2008). First, the microbicidal effector functions of the macrophage are enhanced by increased production of reactive oxygen and nitrogen species by inducing various components of NADPH oxidase and nitric oxide synthase pathways, as well as up-regulation of lysosomal enzymes and production of antimicrobial pepetides and complement components (Schroder et al 2004). As a result, macrophages are able to effectively kill phagocytosed pathogens that might otherwise be resistant to endolysosomal conditions. Second, IFNγ sensitizes macrophages to pathogen recognition by up-regulationg TLRs, inflammasome components, Fc-receptors, and some of their signaling machineries (Zarember et al 2002, Schroder et al et al 2006, O'Mahony et al 2008). Third, antigen presentation is enhanced in classically activated macrophages by up-regulation of MHC- and co-stimulatory molecules as well as by effective antigen processing (Schroder et al 2004). Fourth, what further characterizes the M1 macrophage activation is production of high levels of IL-12 that support a developing Th1 response; production of other pro-inflammatory cytokines (TNFα, IL-1β, IL-6, IL-23); inhibition of

anti-inflammatory cytokine production; and production inflammatory chemokines (CCL2, CCL3, CCL4, IL-8, CXCL9, CXCL10, CXCL11) (Mantovani et al 2004).

5.4.7. Alternative macrophage activation (M2 macrophages)

In addition to classical macrophage activation, macrophages can assume various other functional phenotypes, collectively known as alternative macrophage activation. These alternatively activated macrophages are a heterogeneous and poorly-defined group of cells that participate in a wide range of physiological and pathological processes such as Th2-polarized responses, allergy, parasite immunity, tissue healing, homeostasis, and fibrosis (Mosser and Edwards 2008). The phenotype of these alternatively activated macrophages is perhaps best characterized in relation to classically activated macrophages; the former generally antagonize M1 functions and produce IL-10 instead of IL-12 (Mosser and Edwards 2008). Despite the prevailing confusion in the macrophage nomenclature, the designation "M2 macrophage" is largely reserved for macrophages exposed to IL-4 (Stein et al 1992, Gordon 2003).

IL-4 is produced by various innate immune cells such as eosinophils, mast cells, and basophils and also by activated Th2-lymphocytes. The signals leading to IL-4 production and a subsequent Th2-type of adaptive immune response are complex and only partially understood, but are generally related to detection of parasites and also to tissue injury (Martinez et al 2009, Paul and Zhu 2010).

IL-4 binds to an IL-4 receptor which signals through the JAK-STAT6 pathway, and many of the M2 genes are upregulated directly by STAT6 (Martinez et al 2009, Lawrence and Natoli 2011). The IL-4 receptor also utilizes other less well-characterized signaling pathways that lead to activation of the peroxisome proliferator-activated receptor-γ (PPARγ) and phosphoinositide 3-kinase (PI3K), having a direct effect on the transcription of M2-related genes (Lawrence and Natoli 2011). Importantly, PPARγ also exerts a direct suppressive effect on production of inflammatory cytokines mediated by inhibiting the actions of STAT1, AP-1, and NF-κB (Ricote et al 1998, Pascual et al 2005, Bouhlel et al 2007).

In comparison to classically activated macrophages, M2 polarization is characterized by suppression of pro-inflammatory cytokine production, a suppressed ability to kill intracellular pathogens via diminished production of microbiocidal effector molecules, and suppressed antigen presentation ability, and also by production of high or moderate levels of IL-10 instead of IL-12 (Gordon S 2003, Martinez et al 2009). Membrane receptors with a scavenger function are upregulated, as are a variety of molecules involved in tissue regeneration, wound healing, granuloma formation, and immunity against larger parasites (Martinez et al 2009). For instance, at least in mouse M2 macrophages, the metabolisim of arginine shifts from production of nitric oxide to production of L-ornithine (utilized to produce polyamines) and L-proline (used for cell growth and collagen synthesis) via strong upregulation of the enzyme arginase; this may serve to contain larger parasites but also may be useful during tissue regeneration. Accordingly, in mouse model systems, Th2 response and M2 macrophage activation are crucial in resistance against and the expulsion of parasite infection (Martinez et al 2009). In addition, M2 macrophages secrete a distinct profile of chemokines (CCL13, CCL14, CCL17, CCL18, CCL22, CCL24) specifically recruiting Th2 cells, basophils, and eosinophils (Mantovani et al 2004).

Since the original definition of M2 polarization, several other macrophage phenotypes differing from the M1 or M2 macrophage phenotypes have been described, including macrophages exposed to IL-10, glucocorticoids, TGFβ, and immune complexes

in combination with TLR stimulus. It has been suggested that these macrophages, collectively characterized by high IL-10 production, should be characterized as deactivated or as regulatory macrophages (Mosser and Edwards 2008). In addition to production of IL-10, these macrophage subgroups produce variable amounts of pro-inflammatory cytokines but do not produce ECM components, their characteristic that most clearly distinguishes them from M2 macrophages (Mosser and Edwards 2008).

Moreover, macrophages are readily able to switch their functional status, for example from pro-inflammatory (high IL-12 phenotype) to anti-inflammatory (high IL-10 phenotype) according to the signals derived from their local microenvironment (Stout et al 2005, Murray and Wynn 2011a). Thus, these macrophage polarization states likely reflect a continuum of functional states rather than fixed phenotypes (Mosser and Edwards 2008).

5.4.8. Role of colony-stimulating factors

Mature human macrophages can be maintained and differentiated in vitro by use of either M-CSF or GM-CSF. Although most likely an over-simplification, one suggestion is that macrophages differentiated with M-CSF represent non-activated tissue-resident macrophages while also displaying a clear resemblance to M2 macrophages, and that GM-CSF induces an M1-like phenotype (Verreck et al 2004, 2006, Fleetwood et al 2007, Hamilton 2008). In vivo, M-CSF is secreted under steady state conditions by several cell types, and detectable levels of M-CSF are found in the circulation. In contrast, the production of GM-CSF is local and induced, primarily during inflammation. One suggestion is that systemic levels of M-CSF maintain the general monocyte-macrophage population in the quiescent M0- or M2-like phenotype during the steady state, but GM-CSF, produced by macrophages themselves or by local mesenchymal cells as a response to TLR stimulus, induces the M1-like phenotype during early stages of inflammation (Hamilton 2008).

5.4.9. Osteoclasts and bone resorption

A further example of the diversity of the monocyte macrophage system is the osteoclast, a multinucleated giant cell formed by fusion of precursor cells of the monocyte-macrophage lineage and responsible for resorption of bone tissue during the normal physiological process of bone remodeling and also during pathological bone loss that occurs for example in osteoporosis, rheumatoid arthritis, or indeed, during aseptic loosening (Novack and Teitelbaum 2008).

Bone is a specialized connective tissue composed of a mineralized ECM responsible for mechanical and structural properties of bone, and the group of cells that produce and maintain this matrix (Ross and Pawlina 2011b). In addition to bone-resorbing osteoclasts, bone tissue contains mesenchymal stem cells which differentiate into osteoblasts as instructed by signals from the local microenvironment, for instance bone morphogenetic proteins. Osteoblasts produce and secrete the protein constituents of the bone ECM and also facilitate its mineralization process. When completely surrounded by bone matrix, osteoblasts are termed osteocytes, and they participate in the maintenance of local bone matrix. Ostocytes maintain contact with each other and with bone-lining cells by means of elongated processes that pierce the bone matrix in discrete canaliculi. Bone-lining cells represent both osteoprogenitor cells and osteocytes.

The ECM of bone tissue is composed primarily of type I and type V collagens and to a lesser extent of non-collagenous proteins including proteoglycan macromolecules and associated glycosaminoglycans, multiadhesive glycoproteins (osteonectin, osteopontin), osteocalcin, and various growth factors and cytokines including bone morphogenetic

proteins (Ross and Pawlina 2011b). This complex protein matrix is covered with calcium phosphate in the form of hydroxyapatite crystals.

Adult bone tissue, both compact and spongy bone, is organized into cylindrical osteons comprising concentric lamellae surrounding Volkmann's canal, containing the blood vessels that nourish the cells of the osteon (Ross and Pawlina 2011b). A lamellar structure is created and maintained by a continuous process of bone remodeling in which old bone is first removed by an advancing front of osteoclasts (cutting cone), and the resulting bone defect is filled in by the following front of osteoblasts actively producing and secreting bone-matrix proteins (closing cone) so that the amount of bone removed and produced remains about the same (Raggatt and Partridge 2010, Crockett et al 2011). This process of bone remodeling allows bone tissue to adjust to external stresses and to repair developing fatigue fractures, and is thus imperative for maintaining bone tissues mechanical stability.

For a long time what has been recognized is that bone remodeling and osteoclast function is under tight control by a multitude of regulatory factors including systemic hormones (parathyroid hormone, glucocorticoids, calcitonin, testosterone, estrogen) and locally produced cytokines that either enhance or suppress osteoclast differentiation and function. The exact molecular mechanisms that control osteoclast formation and function have, however, been elucidated only during the last 15 years.

Osteoclast formation and function is primarily controlled by RANKL and its corresponding receptor, receptor activator of nuclear factor kappa B (RANK), expressed on the circulating osteoclast precursors (Boyle et al 2003, Lacey et al 2012). RANKL, a member of the TNF superfamily, is a cell membrane-bound protein that under some conditions can also be solubilized. RANKL is produced by osteoblasts, bone-marrow stromal cells, and by activated lymphocytes (Anderson et al 1997, Wong et al 1997, Fuller et al 1998, Lacey et al 1998, Yasuda et al 1998). The RANKL-RANK system is further regulated by osteoprotegerin (OPG), a soluble decoy receptor for RANKL that inhibits its signaling. OPG is produced in the steady state by osteoblasts and bone marrow stromal cells (Simonet et al 1997). In addition to RANKL, a low level of M-CSF is required for osteoclastogenesis (Tanaka et al 1993).

RANK signaling activates several intracellular signaling cascades including NF-κB, MAP kinases, and AP-1, all of which support osteoclastogenesis and are also induced by TNFα signaling via its type 1 receptor. The most important, and apparently unique property of intracellular RANK signaling is, however, the recruitment of high amounts of TRAF6 adaptor and subsequent downstream activation of transcription factors nuclear factor of activated T cells (NFAT) 1 and NFAT2 (Novack and Teitelbaum 2008). Together with M-CSF-induced signaling through the CSF-1 receptor activating PI3K pathway, which supports osteoclast precursor survival, this complex combination of transcription factors leads to fusion of osteoclast precursors and development of functional osteoclasts (Novack and Teitelbaum 2008).

Bone resorption by osteoclasts is a complex process involving several steps, collectively known as the resorption cycle (Väänänen et al 2000, Teitelbaum 2007, Novack and Teitelbaum 2008). After migration to a resorption site, an osteoclast adheres to bone by a specialized membrane domain known as the sealing zone that circumferentially attaches the osteoclast by its margins to bone matrix. An isolated space known as Howship's Lacuna is thus formed between the osteoclast and underlying bone. Integrin $\alpha_v\beta_3$ is critical for both osteoclast attachment to the bone and for the subsequent cell membrane polarization steps. After attachment, a highly specialized membrane domain called ruffled border is created by the fusion of lysosomal vesicles with the

osteoclast cell membrane facing Howship's Lacuna (Väänänen et al 2000, Teitelbaum 2007, Novack and Teitelbaum 2008). Cytoplasmic carbonic anhydrase II creates carbonic acid and subsequently proton ions that are actively pumped into Howship's lacuna by proton pumps located on the ruffled border. In the low pH of the lacuna, hydroxyapatite crystals are dissolved and the protein matrix of the bone digested by lysosomal proteinases released into the lacuna. The most important of these proteinases is cathepsin K, although MMPs also participate in matrix digestion. The resultant bone-digestion products are removed from the lacuna by transcytosis (Salo et al 1997). After resorption, the osteoclast assumes another cycle of bone resorption or undergoes apoptosis.

In the presence of M-CSF, RANKL is both necessary and sufficient in inducing osteoclast differentiation, whereas OPG inhibits this. Thus the RANK-RANKL-OPG system is the final common pathway that regulates osteoclast formation and function, and further, the local RANKL/OPG ratio is the most important factor regulating bone resorption (Boyle et al 2003, Novack and Teitelbaum 2008, Lacey et al 2012,). The list of additional factors that regulate osteoclast formation, function, and survival is quite extensive, but most of these factors act through modulation of the local RANKL-OPG balance. Once the osteoclast is first formed via the action of M-CSF and RANKL, some factors also have direct effects that support or inhibit osteoclast function.

Importantly, several inflammatory cytokines and chemokines like TNFα, IL-1β, IL-6, IL-7, and CCL2-4 support osteoclast function either directly or indirectly via induction of RANKL and M-CSF expression and suppression of OPG production (Walsh et al 2006, Takayanagi 2009, Koide et al 2010). TNFα appears to play a special role in the regulation of osteoclast formation, as it partially activates signaling cascades similar to those of RANKL, and it is evident that TNFα can induce osteoclastogenesis in the presence of low levels of M-CSF and RANKL (Novack and Teitelbaum 2008). It is of note that the macrophage-polarizing factors IFNγ and IL-4, as well as IL-10, inhibit osteoclastogenesis (Walsh et al 2006, Takayanagi 2009, Koide et al 2011). These inflammation-related mechanisms are probably important in the pathogenesis of inflammatory bone loss associated for example with rheumatoid arthritis and, indeed, also with the development of aseptic loosening.

5.5. Toll-like receptors and other pattern-recognition receptors

5.5.1. Receptor Toll

Receptor Toll was initially discovered as a receptor controlling dorso-ventral axis formation in embryos of *Drosophila melanogaster* (Anderson et al 1985, 1985b). Later researchers recognized that receptor Toll also plays a critical role in the antifungal and antibacterial defense of adult *Drosophila*, because a loss-of-function mutation in the receptors rendered *Drosophila* highly vulnerable to these infections (Lemaitre et al 1996). This observation was in part explained by sequence homology searches which revealed that the cytoplasmic domain of receptor Toll displayed similarities to the cytoplasmic domain of the mammalian IL-1 receptor (Gay and Keith 1991). This suggested that similar signaling pathways leading to production of antimicrobial products were activated by both *Drosophila* Toll and mammalian IL-1-receptor signaling pathways (Gay and Keith 1991). Subsequently, in mice and humans further sequence homology searches revealed a group of transmembrane receptors that had the previously known IL-1 receptor family intracellular signaling domain but a unique Toll-like extracellular domain (Medzhitov et al 1997, Rock et al 1998). Thereafter, one of these Toll-like receptors (TLRs) was proven to be the long-sought specific receptor for the gram-negative bacterial cell membrane

structural component lipopolysaccharide (LPS) that mediates LPS-induced septic shock (Poltorak et al 1998). Together, these findings reveal a novel and critical system by which innate immunity specifically recognizes pathogens and other danger signals. The novelty and importance of this discovery was highlighted by the Nobel Prize in medicine awarded to Bruce Beutler and Jules Hoffmann in 2011 for their pioneering work in the characterization of the TLR system.

5.5.2. TLR structure and function

Currently TLRs have been established as an evolutionary ancient system of PRRs which allow innate immunity to specifically recognize and react to endo- and exogenous danger-signal molecules derived from various infective agents and likely also from sterile tissue damage (Medzhitov and Janeway 1997, Hoffmann et al 1999, Akira et al 2001, Janeway and Medzhitov 2002, Medzhitov 2007). Stimulation of TLRs with the appropriate ligand leads to production of pro-inflammatory and antimicrobial mediators, to production of inflammasome components, and to up-regulation of co-stimulatory molecules in antigen-presenting cells, thus linking the activation of innate and adaptive immunity seamlessly together.

Since the initial discovery of LPS-recognizing TLR4, several other members of this family have been recognized in humans and mice. Currently, the human TLR family contains 10 members, called TLR1-10, and the mouse TLR family 12 members, called TLR1-13. (Akira and Takeda 2004, Akira et al 2006, Beutler 2009, Kawai and Akira 2010). TLR11 to 13 are absent from humans, and TLR10 is non- functional in mice. In humans, TLR1 to 9 are relatively well characterized, but the ligands for and the signaling of TLR10 is currently poorly understood (Table 1). Structurally, TLRs are transmembrane-receptor proteins that consist of three domains, an ectodomain containing leucine-rich repeats that mediate recognition of repeat molecular patterns, a transmembrane membrane-anchoring domain, and an intracellular Toll-interleukin 1 receptor (TIR) domain that initiates and conveys downstream intracellular signaling cascades (Jin and Lee 2008).

TLRs are expressed on a wide variety of innate and adaptive immune cells such as neutrophils, monocyte-macrophages, mast cells, and lymphocytes but also on cells of mesenchymal origin (Akira et al 2006). Based on their cellular location and the type of ligands recognized, TLRs are divided into two broad categories (Akira and Takeda 2004, Akira et al 2006, Beutler 2009, Kawai and Akira 2010). TLR1, TLR2, TLR4, TLR5, TLR6, and TLR10 are localized to the cell membrane and recognize mainly bacterial, fungal, and protozoan structural elements located on external surfaces of these pathogens; these are exposed to TLRs usually by direct cell-to-cell contact or by binding of soluble ligands released from the outer surface of these pathogens into the extracellular space. In contrast, TLR3, TLR7, TLR8, and TLR9 are localized to inner surfaces of intracellular membrane compartments such as endo(lyso)somes and phagosomes, where they recognize bacterial- and viral-derived nucleic acids normally contained within pathogens; these are exposed to TLRs only after lysis of the pathogen by endolysosomal enzymes (Akira and Takeda 2004, Akira et al 2006, Beutler 2009, Kawai and Akira 2010).

Table 1. Human TLRs, their cellular localization, intracellular signaling pathways and activated transcription factors. (*) Pathway exists specifically in plasmacytoid dendritic cells. Data from Kumar et al (2009) and Kawai and Akira (2010). TLR - toll-like receptor; MyD88 - myeloid differentiation factor 88; TRIF - TIR domain–containing adaptor inducing interferon-β; NF-κB - nuclear factor kappa-light-chain-enhancer of activated B cells; MAPK - mitogen-activated protein kinases; IRF - interferon regulatory factor.

Receptor	Localization	Pathway	Transcription factors	Mediators produced
TLR1	Cell membrane	MyD88	NF-κB, IRF5, MAPKs	Inflammatory cytokines
TLR2	Cell membrane	MyD88	NF-κB, IRF5, MAPKs	Inflammatory cytokines
TLR3	Endosome	TRIF	NF-κB, IRF5, MAPKs IRF3	Inflammatory cytokines Type 1 interferons
TLR4	Cell membrane	MyD88 TRIF	NF-κB, IRF5, MAPKs IRF3	Inflammatory cytokines Type 1 interferons
TLR5	Cell membrane	MyD88	NF-κB, IRF5, MAPKs	Inflammatory cytokines
TLR6	Cell membrane	MyD88	NF-κB, IRF5, MAPKs	Inflammatory cytokines
TLR7	Endosome	MyD88	NF-κB, IRF5, MAPKs IRF7*	Inflammatory cytokines Type 1 interferons*
TLR8	Endosome	MyD88	NF-κB, IRF5, MAPKs	Inflammatory cytokines
TLR9	Endosome	MyD88	NF-κB, IRF5, MAPKs IRF7*	Inflammatory cytokines Type 1 interferons*
TLR10	Cell membrane	MyD88	NF-κB, IRF5, MAPKs	Inflammatory cytokines

5.5.3. PAMPs, alarmins, and DAMPs

Exogenous, or foreign, ligands for TLRs include a wide range of bacterial-, viral-, fungal- and protozoa-derived biomolecules such as lipids, lipoproteins, carbohydrates, peptides, and nucleic acids collectively designated as pathogen-associated molecular patterns (PAMPs, Table 2) (Janeway 1989, Janeway and Medzhitov 2002). Reflecting the system's old phylogenetic age, PAMPs are typically well-conserved and fundamental structural components of pathogens, and, as their name implies, typically have a polymeric and repeating molecular structure (Medzhitov and Janeway 1997, Hoffmann et al 1999, Akira et al 2006). These typical PAMP characteristics are exemplified by four TLR ligands: LPS and LTA, fundamental cell-membrane structural liposaccharides of gram-negative and gram-positive bacteria recognized by TLR4 and TLR2 respectively, double-stranded RNA typically originating from viruses and recognized by TLR3, and bacterial CpG DNA recognized by TLR9 (Poltorak et al 1998, Yoshimura et al 1999, Alexopoulou et al 2001, Hemmi et al 2000).

In addition to these exogenous TLR ligands signaling tissue infection by foreign pathogens, endogenous TLR ligands have also been identified (Table 2) (Akira and Takeda 2004, Rifkin et al 2005, Wagner 2006, Miyake 2007, Kono and Rock 2008, Kawai and Akira 2010). These endogenous TLR ligands, collectively known as "alarmins" in reference to their ability to alarm the innate immune system of sterile-tissue damage, are host molecules that are normally securely contained in healthy tissues; they are exposed to TLR recognition only after release from their restricted physiological compartments by cell- and tissue damage (Bianchi 2007, Kono and Rock 2008). Once released, alamins can bind to their corresponding TLRs and other PRRs of the innate immunity and thus initiate an inflammation reaction and subsequent activation of the adaptive immune system. Typical sources of molecules that can function as alarmins include necrotic cells and

damaged ECM (Bianchi 2007, Kono and Rock 2008). During physiological processes like cell apoptosis and subsequent rapid clearance of apoptotic bodies by tissue-resident macrophages, or during normal ECM turnover, no alarmin molecules are released, and thus the innate immune system can effectively sense sterile tissue damage and further distinguish it from normal apoptotic clearance of senescent or pre-malignant cells (Kono and Rock 2008, Kawai and Akira 2010).

Several molecules normally contained in the intracellular compartment and released during cell necrosis can function as alarmins (Bianchi 2007, Kono and Rock 2008, Kawai and Akira 2010). For example, uric acid, a physiological end-product of purine base metabolism, is physiologically present in relatively high concentrations in cell cytoplasm. Once released during cell necrosis, uric acid tends to form crystals due to the high sodium concentration of extracellular space. These monosodium urate microcrystals are ligands for TLR2 and NALP3 inflammasome, and their formation and recognition by innate immune cells are also the underlying mechanisms of gout arthritis (Shi et al 2003, Liu-Bryan et al 2005, Martinon et al 2006). In addition to uric acid, several other intracellular components such as heat-shock proteins (HSPs) can function as alarmins via recognition by TLR2 or TLR4 (Kono and Rock 2008). Some of the intracellular alarmins such as the high-mobility group box 1 (HMGB1) can also be released from activated innate immune cells, allowing these alarmins to function in a cytokine-like manner, further adding to the complexity of alarmin signaling (Scaffidi et al 2003).

During sterile tissue damage, ECM can be damaged and degraded either directly due to the underlying tissue insult or indirectly via the action of various proteases released from either necrotic cells or invading and activated innate immune cells (Kono and Rock 2008, Kawai and Akira 2010). Several components of the fragmented ECM such as biglycan, hyaluronic acid, versican, and fibronectin extradomain A can function as alarmins by ligation of TLR2 or TLR4 (Okamura et al 2001, Jiang et al 2005, Schaefer et al 2005, Kim et al 2009).

Since the description of the first endogenous TLR ligands, a vigorous debate has arisen over their actual nature and their significance for the immune system. Some authors have demonstrated that the inflammatory and TLR2- or TLR4-stimulating properties of some alarming molecules are actually due to minute bits of LPS contamination. Thus an alternative to the endogenous TLR ligand hypothesis is that during infection and inflammation, alarmin molecules serve as PAMP-binding and -presenting molecules that improve the sensitivity of PAMP detection during tissue infection, rather than being actual endogenous TLR ligands themselves (Tsan and Gao 2004, Erridge 2010). Some alarmin molecules, however, clearly possess TLR-stimulating activity independent of PAMP contamination, and because it undeniably makes sense that the immune system is also able to sense sterile tissue damage, the generally accepted view is that actual endogenous TLR ligands do exist (Seong and Matzinger 2004, Matzinger 2007, Kono and Rock 2008, Kawai and Akira 2010). Collectively, exogenous PAMPs and endogenous alarmins are referred to as danger-associated molecular patterns (DAMPs), called this to refer to a larger shift in immunological paradigm. According to this postulated “danger model,” the immune system in general is more interested in detecting and clearing what is potentially dangerous than in discriminating between self and non-self (Matzinger 1994, 2002). Danger signals can be derived both from exogenous (e.g. bacteria) or endogenous sources (e.g. necrotic cells), and both of these share common properties absent under physiological conditions, for example, exposed hydrophobicity or extracellular RNA and DNA (Matzinger 2002, 2007, Seong and Matzinger 2004).

Table 2. Examples of endo- and exogenous TLR ligands. Data from Akira and Takeda (2004), Akira et al (2006), Kono and Rock (2008), Kumar et al (2009), and Erridge (2010). HSP - heat-shock protein; ECM - extracellular matrix; HMGB1 - high-mobility group box 1; ssRNA - single stranded RNA; dsRNA - double stranded RNA.

TLR	PAMP	Source	Alarmin	Source
TLR1/2	Triacyl lipopeptides	Bacteria Mycobacteria		
TLR2	Peptidoglycan	$Gram^+$ bacteria	HSPs	Necrotic cells
	Lipoteichoic acid	$Gram^+$ bacteria	HMGB1	Necrotic cells
	Porins	*Neisseria*	Uric acid	
	Lipoarabinomannan	Mycobacteria	Biglycan	ECM
	Zymosan	Fungi	Hyaluronan	ECM
	Phospholipomannan	*Candica albicans*	Versican	ECM
TLR3	ssRNA, dsRNA	Viruses	RNA	Necrotic cells
TLR4	Lipopolysaccharide	$Gram^-$ bacteria	HSPs	Necrotic cells
	Mannan	*Candida*	HMGB1	Necrotic cells
	Envelope proteins	Viruses	Uric acid	Necrotic cells
			Biglycan	ECM
			Hyaluronan	ECM
			Heparan sulfate	ECM
			Tenascin-C	ECM
			Fibrinogen	ECM
			Fibronecting	ECM
			Surfactant protein A	ECM
			β-defensin	Macrophages
TLR5	Flagellin	Flagellated bacteria		
TLR6/2	Diacyl lipopeptides	Mycoplasma		
	Lipoteichoic acid	$Gram^+$ bacteria		
	Zymosan	Fungi		
TLR7	ssRNA	Viruses	RNA	Necrotic cells
TLR8	ssRNA	Viruses	RNA	Necrotic cells
TLR9	dsDNA, CpG-DNA	Viruses Bacteria	DNA	Necrotic cells

5.5.4. TLR signaling

After binding of their corresponding ligands, TLRs form receptor dimers which initiate intracellular signal transduction cascades by conformational changes and subsequent recruitment of several adaptor molecules to their intracellular TIR domain (Akira and Takeda 2004, Akira et al 2006, Kumar et al 2009, Kawai and Akira 2010). Most TLRs function as homodimers, but TLR2 typically forms receptor heterodimers with TLR1, TLR6, and TLR10, thus expanding the possible ligand specificities of these particular receptors. For instance, due to subtle differences in the molecular structure of TLR1 and TLR6 ectodomains, the TLR2-TLR1 heterodimer recognizes triacylated lipopeptides, while the TLR2-TLR6 dimer recognizes diacylated lipopeptides (Jim and Lee 2008). TLRs also utilize co-receptors. The best known example is LPS recognition by

TLR4, where soluble LPS is first bound to LPS-binding protein present in the circulation. Next the LPS-binding protein complex is bound to cell-surface CD14, which then delivers the LPS complex to TLR4 that functions in a complex with additional co-receptor, myeloid differentiation protein 2 (Kawai and Akira 2010).

TLR-ligand interaction and subsequent receptor dimerisation initiate intracellular signaling by recruitment of various adaptor molecules to the receptor's TIR domain. Currently, four different TIR domain-containing adaptor proteins have been recognized: myeloid differentiation factor 88 (MyD88), a MyD88 adaptor-like (Mal), a TIR domain-containing adaptor inducing IFN-β (TRIF), and a TRIF-related adaptor molecule (TRAM). MyD88 is recruited by all TLRs except TLR3, and most TLRs recruit MyD88 directly to their TIR domain; TLR2 and TLR4, however, use MAL as an additional adaptor between the TIR domain and MyD88. TLR3 recruits TRIF directly, whereas TLR4 uses TRAM as an additional adaptor between the TIR domain and TRIF. The signals derived from these various adaptor molecules thus converge into two primary intracellular signaling pathways: MyD88-dependent and TRIF-dependent pathways (Akira and Takeda 2004, Akira et al 2006, Kumar et al 2009, Kawai and Akira 2010). The major difference between these two signaling pathways, in addition to different mediators of the intracellular-signaling intermediate steps, is whether the pathway leads to production of type I interferons.

Except for TLR3, all TLRs use, either directly or via adaptor MAL, the MyD88-dependent signaling pathway which leads, via several intermediate steps, to activation of inflammatory transcription factors IRF5, MAP kinases, AP-1, and NF-κB, resulting in an inflammatory reaction mediated by production of various inflammatory chemokines, cytokines, growth factors, inflammasome components, and costimulatory molecules (Akira and Takeda 2004, Akira et al 2006, Kumar et al 2009, Kawai and Akira 2010).

The TRIF-dependent signaling pathway is utilized exclusively by TLR3 and partly, via adaptor TRAM, by TLR4, which uses also the MyD88-dependent signaling pathway. The TRIF-dependent pathway leads, via intermediate steps, to activation of MAPKs and NF-κB but also to activation of transcription factor IRF3, leading to production both of inflammatory cytokines and of type I interferons—in macrophages, these typically being IFNβ (Akira and Takeda 2004, Akira et al 2006, Kumar et al 2009, Kawai and Akira 2010).

There also exist cell-type-specific differences in TLR signaling allowing further elaboration of DAMP-induced cellular responses. The best characterized example of this cell-type-specific TLR signaling is plasmacytoid dendritic cells, which apparently exist to proficiently sense and repel viral infections. In plasmacytoid dendritic cells, TLR7 and TLR9 use a non-conventional MyD88-dependent signaling pathway, activation of which leads, in addition to traditional activation of MAPKs and NF-κB, to the activation of transcription factor IRF7 and subsequent production of type I interferons, typically both IFNα and IFNβ (Akira and Takeda 2004, Akira et al 2006, Kumar et al 2009, Kawai and Akira 2010).

5.5.5. Regulation of TLR signaling

Unlimited and overt inflammatory responses are potentially detrimental and even lethal to the host, as exemplified by various autoimmunity disorders and septic shock. As the TLR system, along with other PRRs, is largely responsible for the initial sensing of danger and initial activation of both the innate and adaptive arms of the immune system, it is not surprising that multiple levels of TLR signaling regulation exist (Liew 2005, Lang and Mansell 2007, Kawai and Akira 2010). These regulatory mechanisms both restrict

initial TLR activation and, after successful TLR signaling, shut down the signal so that collateral damage to host tissues is limited, and tissue repair and regeneration processes can commence.

The several potential mechanisms of TLR signaling regulation recognized include production of soluble decoy receptors (thus far recognized for TLR2 and TLR4), splice variants of TLR intracellular signaling adaptors, TLR degradation via ubiquitination, and overt TLR signaling-induced apoptosis (Liew 2005, Lang and Mansell 2007, Kawai and Akira 2010). Additionally, intracellular TLR-signaling pathways include several proteins that bind to and inhibit different levels of TLR-signaling cascades (Liew 2005). Some of these (PI3K, TOLLIP, TRAILR) are constitutively expressed in monocyte-macrophages and limit TLR signaling in a continuous manner, thus creating in-built resistance, or a threshold to be overcome by TLR signaling, while the expression of others (TANK, IRAKM, SOCS, A20) is regulated to accommodate to prevailing conditions.

Activation of TLR signaling with a appropriate ligand typically leads, in addition to the pro-inflammatory response, also to production of anti-inflammaotry mediators and reciprocal up-regulation of inhibitory signaling mediators and suppression TLR signaling (Liew 2005, Lang and Mansell 2007, Kawai and Akira 2010). The best-known example of these negative feedback-loop mechanisms typical for TLR signaling is endotoxin tolerance; LPS-treated macrophages become ractory to subsequent LPS treatments and in response to a repeated LPS stimulus produce quantities of IL-10 instead of pro-inflammatory cytokines (Biswas and Lopez-Collazo 2009). The underlying mechanisms of this phenomenon are complex and include suppression of TLR4 signaling on multiple levels (Biswas and Lopez-Collazo 2009).

In addition to signaling-induced negative feed-back inhibition, TLR signaling is regulated by several factors including the local cytokine microenvironment and systemic hormones that typically have broad effects on many levels of TLR signaling and can influence TLR expression and degradation levels directly, regulate the amounts of their intracellular signaling inhibitors or directly activate/repress TLR-induced transcription factors (Liew 2005, Lang and Mansell 2007, Kawai and Akira 2010). Indeed, a concept of a tissue-specific or tissue-regulated immune reaction has been recently introduced (Matzinger 2007, Matzinger and Kamala 2011). One premise of this model is that signals derived from the local microenvironment determine the tissue-specific threshold for TLR signaling. For example, due to the physiologically prevailing strong anti-inflammatory milieu, the innate immune cells residing in the wall of the gut do not react to the normal flora despite the abundance of potential PAMPs.

In general, pro-inflammatory cytokines up-regulate TLR expression and promote TLR signaling, whereas anti-inflammatory cytokines, especially IL-10, effectively down-regulate TLR expression and suppress their signaling by elevating the amounts of their intracellular signaling inhibitors (Liew 2005, Lang and Mansell 2007, Kawai and Akira 2010). Among systemic hormones, the effects of glucocorticoids on TLR signaling are those most widely studied (Chinenov and Rogatsky 2007). Not surprisingly, glucocorticoids suppress several layers of TLR signaling by raising the amounts of their intracellular signaling inhibitors and directly interacting with TLR-activated transcription factors.

5.5.6. TLRs and macrophage polarization

Macrophage polarization has profound effects on TLR expression and signaling. IFNγ priming enhances TLR expression and signaling, and, accordingly, IFNγ treatment effectively sensitizes M1 macrophages to TLR ligands; the expression of some TLR-

regulated genes requires this IFNγ priming, while the dose-response curve of other genes is shifted by the IFNγ treatment so that lower concentrations of TLR ligand are sufficient to cause gene reading (Schroder el al 2006, Hu et al 2008). The negative feedback loops typical of TLR signaling are also suppressed and, for example, IFNγ treatment eliminates endotoxin tolerance (Adib-Conquy et al 2002, Bosisio et al 2002, Chen et al 2010). This enhancement of TLR signaling on M1 macrophages is the result of the complex cross-talk between IFNγ and TLR signaling pathways (Schroder el al 2006, Hu et al 2008). For example, the levels of TLRs, their co-receptors, and various intracellular signaling components are directly up-regulated, and inhibitory signaling mediators down-regulated by IFNγ and the accompanying activation of STAT1 (Muzio et al 2000 and 2001, Bosisio et al 2002, Zarember et al 2002, Tamai 2003, Radstake et al 2004, O'Mahony et al 2008). Additionally, IFNγ-induced STAT1 and TLR signaling-activated transcription factors, like NF-κB, synergize by binding to promoter-regions of the same target genes (Schroder el al 2006). Furthermore, TLR signaling pathways reciprocally enhance IFNγ signaling by partially activating STAT1 via MAP kinase pathways (Schroder el al 2006). Finally, IFNγ suppresses TLR-signaling-induced production of IL-10 (Hu et al 2006, 2008).

Regulation of TLR signaling in M2 macrophages is less well understood, but it appears that in M2 macrophages, TLR signaling is generally inhibited, possibly by general inhibition of inflammatory signaling pathways partly mediated by PPARγ, by direct down-regulation of TLR expression, or by increased production of IL-10 (Staege et al 2000, Lawrence and Natoli 2011). A possibly analogous phenomenon of TLR-signaling up-regulation via Th1-related cytokines and down-regulation by Th2-related cytokines also occurs in various other cell types (Faure et al 2001, Wolfs et al 2002, Mueller et al 2006, Romieu-Mourez et al 2009). Enhancement of TLR signaling in M1 macrophages and suppression of TLR signaling in M2 macrophages likely reflect the natural course of an inflammation-resolution-healing sequence and a corresponding switch in macrophage phenotype from inflammatory M1 to M2 macrophages that promote tissue regeneration and healing.

5.5.7. Other PRRs, inflammasome, and IL-1β

Since the discovery of TLRs, at least three additional PRR families have been identified (Medzhitov 2007, Kawai and Akira 2010). C-type lectin-like receptors (such as the mannose receptor) are localized to cell membrane and participate in microbial recognition and a subsequent inflammatory reaction via NF-κB activation. NLR receptors (divided into NOD-like receptors and NALPs) and RIG-I-like receptors are large families of cytoplasmic PRRs. Although the detailed mechanisms of DAMP recognition and subsequent signaling by these various receptors are still under investigation, these cytosolic PRRs may participate in recognition of various danger signals located in the cytoplasmic compartment, including viral and bacterial nucleic acids and toxins. Signaling through these receptors leads to NF-κB activation but also to production if type I interferons (Medzhitov 2007, Kawai and Akira 2010).

Importantly, NALP activation is required for the assembly of an inflammasome, a large cytoplasmic multiprotein complex responsible for activation of caspase-1, which is required for secretion of the strong inflammatory mediators IL-1β and IL-18 (Davis et al 2011, Strowig et al 2012). Inflammasome assembly is initiated by a large repertoire of DAMPs. In contrast to TLRs, NALPs may sense more general cellular stress such as generation of reactive oxygene species, rather than recognizing specific DAMPs. In the context of wear-debris recognition and induced responses, it is noteworthy that several phagocytosed crystalline structures (uric acid, cholesterol, hydroxyapatite crystals) as well

as inhaled foreign bodies (asbestos fibers, silica) can activate NALP3 inflammasome via endosomal damage and release of endo(lyso)somal constituents into the macrophage cytoplasm (Davis et al 2011, Strowig et al 2012).

6. Aims of the study

Wear-particle-induced macrophage activation to the inflammatory phenotype is considered a fundamental event in the pathogenesis of aseptic loosening, and the sequence of events leading from macrophage activation to osteolysis is well characterized.The exact mechanisms by which biomaterial particles are recognized by macrophages and how their phagocytosis leads to macrophage activation have, however, remained elusive. Recent observations are that wear particles of various natures are relatively inert, cause only limited macrophage activation and osteolysis, and have their inflammatory properties largely dependent on bacterial structural components adhering to their surfaces. This led to the hypothesis that recognition of bacterial product-coated wear particles and subsequent activation of interface tissue macrophages may be mediated by TLRs. In addition, as immunological research indicates that the expression of TLRs and macrophages' ability to produce inflammatory mediators is largely dependent on macrophage polarization, we further hypothesized that macrophage polarization may be an important determinant of the way that macrophages react to wear particles.

Accordingly, the specific aims of this study were

1. To characterize the possible presence of TLR1-9 in the aseptic interface tissue.

2. To compare the inflammatory cell profile and localization of TLR4 and TLR9 between septic and aseptic interface tissues.

3. To discover whether wear particles directly regulate TLR levels in a mouse model of wear-particle-induced inflammation and in a macrophage culture system.

4. To discover whether macrophage polarization affects macrophage wear-particle responses.

7. Materials and methods

7.1. Patients and samples (I, II, IV)

7.1.1. Ethical considerations

The ethics committees of Helsinki University Central Hospital (I, II, IV) and of the Rizzoli Orthopaedic Institute (II) approved the use of patient samples in studies of this thesis.

7.1.2. Controls (I, II)

Samples of hip joint synovial tissue were came from primary total joint replacement operations performed for clinically and radiologically diagnosed primary osteoarthritis of the hip joint. Patients with secondary osteoarthritis and rheumatoid arthritis were excluded on the basis of patient history, clinical symptoms, radiological examination, and laboratory data. Five patients (one man, four women, mean age 73.6) undergoing primary hip arthroplasty were included in Study I, and five patients (one man, four women, mean age 74.4) in Study II. Additionally, ten patients (four men, six women, mean age 67.2) were included in the TLR-expression profiling study (Pajarinen J, Jämsen E, Konttinen YT, unpublished results)

7.1.3. Patients with aseptic THR loosening (I-II)

Tissue samples of synovial membrane-like interface tissue were obtained from total hip joint revision operations performed for aseptic loosening of the THR components. Initial diagnosis of aseptic loosening was by clinical and radiological evaluation and was later confirmed by negative intraoperative bacterial cultures and also by typical histology. Four patients with aseptic THR loosening (two men, two women, mean age 70.5) were included in Study I, and five (two men, three women, mean age 71.2) in Study II. Additionally, twelve patients (three men, nine women, mean age 67.9) were included in the TLR-expression profiling study (Pajarinen J, Jämsen E, Konttinen YT, unpublished results)

7.1.4. Patients with septic THR loosening (II)

Tissue samples of peri-implant tissue came from total hip joint revision operations performed for septic THR loosening. Initial diagnosis of septic THR loosening was by clinical, laboratory, and radiological evaluation later confirmed by positive intraoperative bacterial cultures. Ten patients (two men, eight women, mean age 61.2) with THR infection were included in Study II. In five cases, the implant was infected by *Staphylococcus epidermidis*, in four cases by *Propionibacterium acnes*, and in one case by alpha-hemolytic *Streptococcus*.

7.1.5. Tissue processing (I, II)

Immediately after collection, all tissue samples intended for immunohistochemistry were fixed in 30% formaldehyde for 24 to 48 h, dehydrated in ethanol, cleared in xylene, and embedded in paraffin. Tissue sections 5 to 8 µm thick were then cut with a microtome, deparafinized in xylene, and rehydrated in a decreasing ethanol series. For examination of general tissue architecture, tissue sections were stained with standard hematoxylin-eosin and then further analyzed with immunohistochemistry.

For the unpublished TLR-expression profiling study, tissue samples were immediately after collection snap-frozen in isopentane, precooled in dry ice, and stored at -75 ºC until use. About 0.1-mg tissue pieces were cut, with the tissue continuously frozen by dry ice, and total RNA was isolated from these tissue pieces with Trizol reagent (Invitrogen, Carlsbad, CA, USA) and a Ultra-Turrax tissue homogenizer (Janke & Kunkel, IKA-Laborthecnik), followed by RNA purification using RNeasy Mini Kit (Qiagen, Düsseldorf, Germany). Total RNA was stored at -75ºC until use.

7.1.6. Cell samples (III, IV)

For the particle stimulations of Study III, the mouse monocyte/macrophage cell line RAW 264.7 was bought from ATCC (Manassas, Virginia, USA). For human peripheral blood monocyte isolation (PBMC) and monocyte-macrophage differentiation and particle stimulation studies in Study IV, buffy coats of four healthy blood donors were obtained from the Finnish Red Cross Blood Service.

7.2. Titanium particle preparation (III, IV)

Titanium particles, mean diameter 3.7 +/- 1.8 μm as determined by scanning electron microscopy, were bought from Alfa Aesar (Ward Hill, MA, USA). To minimize any possible LPS contamination, the particles were washed with alternating cycles of 20-hour incubation in 25% nitric acid and 0.1 N NaOH in 95% ethanol (Ragab et al 1999). Between these treatments, particles were washed twice in cell-culture-grade PBS. After five cycles of washes, particles were resuspended in PBS and stored at +4 ºC until use. After washes, particle LPS levels were determined by Limulus amebocyte lysate chromogenic endpoint assay kit. Study III used a kit with a minimum LPS detection limit of 0.125 EU/ml (BioWhittaker, Walkersville, MD, USA) and Study IV a kit with a minimum LPS detection limit of 0.01 EU/ml (Hycult Biotech, Uden, the Netherlands). In both instances, particle LPS levels were below these assays' detection limits. For in vitro macrophage stimulation experiments, particles were diluted in cell culture medium to obtain the indicated concentrations and for the in vivo particle-induced inflammation model, particles were diluted in carrier solution consisting of 8.5 mg/ml sodium hyaluronate in PBS.

7.3. In vivo model of particle-induced inflammation (III)

A mouse model of particle-induced inflammation was produced at Stanford University (Orthopedic Research Laboratory, Stanford, CA, USA). Briefly, 14 adult (12- to 14-week-old) male C57BL/6 WT mice were obtained from the Stanford University in-house breeding colony. Institutional guidelines for the care and use of laboratory animals were strictly followed. Animals were anesthetized with an intraperitoneal injection of a 1:1 ketamine/xylazine, after which the distal femur was accessed through a medial parapatellar arthrotomy. A 27-gauge needle was used to penetrate the intercondylar notch to gain access to the medullary cavity of the femur. A 25-gauge needle was then drilled into the femur, after which 1.39×10^8 titanium particles in 100 μl of carrier solution were injected into the intramedullary canal in the right femur, but the carrier solution without particles was injected into the intramedullary canal of the left femur. Kirschner wires of 25-gauge stainless steel (McMaster-Carr, Chicago, IL, USA), cut into 10-mm rods, cleaned, and steam autoclaved, were then inserted bilaterally into both femoral canals.

Seven animals each were killed at 2 or 10 weeks postoperatively. Distal femurs were cut, fixed in 10% neutral buffered formalin (Sigma-Aldrich, St. Louis, MO, USA) for 24 h at 48ºC, and decalcified in 23% formic acid for 48 h at room temperature. The stainless-steel rod was carefully extracted before the samples were embedded in paraffin. Beginning at 5 mm from the knee joint, 5-µm transverse sections were cut from the distal femur, deparafinized in xylene, and rehydrated in decreasing ethanol series. For the examination of general tissue architecture, tissue sections underwent routine hematoxylin-eosin staining, andTLRs were analyzed at both time points with immunohistochemistry.

7.4. Immunohistochemistry (I, II, III)

Immunohistochemical evaluation of aseptic and septic interface tissues and control synovial tissues as well as of mice femurs was done either manually (for TLRs, in Studies I, III) or by an automated immunohistochemial staining robot (for cell type markers, Study II). For both the manual and automated protocols, antigen retrieval was first performed on deparaffinized and rehydrated tissue sections at 98ºC in 10 mM citrate buffer, pH 6.0, for 24 min in Milestone Mega T/ T microwave oven (Milestone s.R.L., Sorisole, Italy) and by a specific antigen retrieval program, followed by cooling down at room temperature for 20 min and washing with phosphate-buffered saline (PBS, pH 7.4). Neutrophil elastase staining required no antigen retrieval (Study II).

For manual staining of TLRs, tissue sections were then treated serially with 1) 3% H_2O_2-methanol for 20 min; 2) 10% normal goat serum (Vector Laboratories, Burlingame, CA, USA) diluted in 0.1% bovine serum albumin (BSA) in PBS for 1 h; 3) One of the polyclonal, affinity-purified rabbit anti-human IgG antibodies specific for givenTLR and displaying cross-reactivity to corresponding mouse TLRs (Table 3), diluted in 0.1% BSA-PBS, overnight at 4 ºC;) Biotinylated goat anti-rabbit IgG (Vector Laboratories) diluted in 0.1% BSA-PBS for 1 h; 5) Avidin-Biotin-peroxidase complex (Vector)for 1 h, and finally, 6) H_2O_2-3,3-diaminobenzidine tetrahydrochloride (DAKO A/S, Glostrup, Denmark) for 5 min, followed by washing in distilled water. Between steps 1 and 2 and 3, 4, and 5, the sections were washed for 3x5 min in PBS. The specificity of the staining was confirmed by including negative controls stained with normal rabbit IgG (Jackson Immunoresearch, West Grove, PA, USA) at the same concentration as the highest specific primary rabbit antibody (2.7 µg/mL).All the incubations were done at room temperature and in humidified chambers unless otherwise stated.

Immunohistochemical staining of cell type-specific markers was done by the MSIP protocol of the DAKO TechMate Horizon Immunostainer robot (DAKO A/S) and the ChemMate LSAB staining kit (DAKO A/S), by manufacturer-provided protocols. Sections were serially treated with 1) primary antibodies specific for given cell type marker (Table 4) diluted in manufacturer-provided antibody diluent buffer (DAKO) for 25 min; 2) biotinylated anti-rabbit or anti-mouse secondary antibodies in diluent buffer for 25 min; 3) peroxidase blocking solution (DAKO) for 3x3 min; 4) peroxidase-conjugated streptavidin for 25 min, and finally with 5) H_2O_2-3,3-diaminobenzidine tetrahydrochloride (DAKO A/S) for 5 min. Between the steps, sections were washed withChemMate washing buffer for 3x5 min and dried with absorbent pads. All incubations were done at room temperature. Specificity of the staining was confirmed by including negative controls stained with irrelevant IgGs of the same subclass and used in similar concentration, as the specific cell-marker antibodies (mouse control antibodies from DAKO, normal rabbit IgG from Jackson Immunoresearch).

After both the manual and automated immunohistochemical staining, sections were counterstained in hematoxylin for 5 s, washed with running tap water for 5 min, dehydrated in increasing ethanol series, cleared in xylene, and mounted in mounting medium.

Stained tissue sections were examined under, and photographed with a Leitz Diaplan microscope (Wild Leitz, Wetzlar, Germany) coupled to a 5MP digital DFC420 camera and Application Suite 3.0 image analysis system (both from Leica Microsystems, Wetzlar, Germany). The number of TLR-positive cells was calculated in a representative high power field (I) or the number of TLR and specific cell marker-positive cells was evaluated on a semi-quantitative scale (II, III): no positive staining (-); occasional positive cells (±); some positive cells (+); moderate numbers of positive cells (++); and large numbers of positive cells (+++), by two observers.

Table 3. Dilutions of anti-TLR primary antibodies in the studies. All Polyclonal rabbit IgG and from Santa Cruz Biotechnology (Santa Cruz, CA, USA). (*)Dilution used in Study III. TLR - toll-like receptor

Antibody	Dilution	Study
TLR1	0.8/0.5* µg/ml,	I, III
TLR2	2.7/1.3* µg/ml	I, III
TLR3	2.0 µg/ml	I
TLR4	1.3/1.3* µg/ml	I, II, III
TLR5	0.8/1.3* µg/ml	I, III
TLR6	1.0 µg/ml	I
TLR7	0.8 µg/ml	I
TLR8	2.7/1.3* µg/ml	I, III
TLR9	0.5/0.5 µg/ml	I, II, III

Table 4. Details of primary antibodies to identify differing cell populations in aseptic, septic, and control synovial, and peri-implant tissues (Study II). (1) Novocastra, Newcastle, UK, (2) Stressgen, Victoria, Canada, (3) Original concentration of the CD163 antibody was lacking with only the working antibody dilution provided. CD - cluster of differentiation; NE - neutrophil elastase; HSP - heat-shock protein; Ig - immunoglobulin.

Antibody	Cell type	From	Type	Dilution
CD3	T lymphocytes	DAKO	Polyclonal rabbit IgG	5 µg/ml
CD20	B lymphocytes	DAKO	Monoclonal mouse $IgG_{2a\kappa}$	0.7 µg/ml
CD68	Macrophages	DAKO	Monoclonal mouse $IgG_{1\kappa}$	0.85 µg/ml
CD138	Plasma cells	Novocastra[1]	Monoclonal mouse $IgG_{1\kappa}$	1.25 µg/ml
CD163	Macrophages	Novocastra	Monoclonal mouse $IgG_{1\kappa}$	1/50[3]
NE	Neutrophils	DAKO	Monoclonal mouse $IgG_{1\kappa}$	0.65 µg/ml
HSP47	Fibroblasts	Sressgen[2]	Monoclonal mouse IgG_{2b}	1.0 µg/ml

7.5. Cell cultures (III, IV)

7.5.1. Mouse macrophage particle stimulation (III)

Mouse monocyte/macrophage cell line RAW 264.7 was cultured in high glucose DMEM (Life Technologies, Grand Island, NY, USA) with 10% FCS and 1% antibiotics in humidified 5% CO_2-in-air in a +37°C incubator. Cells were refreshed twice a week, split 1:10 with scraping once a week, counted with aZ1 Coulter Particle Counter (Beckman Coulter, Indianapolis, IN, USA) and used in the experiments at passages 8 to 10. For particle stimulations, cells were transferred to 6-well plates (BD, Franklin Lakes, NJ, USA), $2x10^5$ cells per well, in 2 ml of medium. Cells were allowed to adhere for 1 to 2 h, after which the medium of the particle-stimulated cell group was changed to 2 ml of medium supplemented with approximately $1x10^7$ particles, equaling about 100 particles per cell. Corresponding control cells received only culture medium. After 0, 2, 6, 10, and 24 hours of particle stimulation, the medium was removed and total RNA extracted with Trizoll reagent (Invitrogen), and stored at -75 °C until used for quantitative real time-PCR analysis (qRT-PCR) of TLR mRNA expression.

7.5.2. Human monocyte isolation and differentiation (IV)

PBMC were isolated from buffy coats of healthy blood donors by the Ficoll-Paque PLUS (GE Healthcare, Piscataway, NJ, USA) density gradient method. To exclude lymphocytes from the cell culture model, monocytes were further purified from other PBMC populations by $CD14^+$ positive selection using the MACS system (Miltenyi Biotech, Bergisch Gladbach, Germany). The number of the $CD14^+$ cells obtained was counted with a Z1 Particle Counter (Beckman Coulter), after which cells were divided into tissue-culture-treated 24-well plates (BD), $3x10^5$ cells /well in 0.5 ml of Gibco RPMI-1640 GlutaMAX-1 medium (Life Technologies) supplemented with 10% fetal bovine serum (FBS) and penicillin-streptomycin antibiotic cocktail (complete medium). Cells were allowed to adhere for 1 hour in an incubator, after which non-adherent cells were removed by a gentle rinse with PBS. The remaining adherent monocytes were differentiated into mature M0 macrophages by culturing them for 7 days in complete medium supplemented with 100 ng/ml of macrophage-colony-stimulating factor (M-CSF, R&D systems, Minneapolis, MN, USA). Cells were maintained in a humidified, 5% CO_2-in-air, +37 °C incubator and refreshed once during the 7-day differentiation.

7.5.3. Induction of macrophage polarization (IV)

Following macrophage differentiation, macrophages were polarized by 1-day culture in a complete medium supplemented with 100 ng/ml of M-CSF (M0 polarization), 20 ng/ml IFNγ (M1 polarization), or 20 ng/ml IL-4 (M2 polarization) (all from R&D systems).

7.5.4. Particle stimulation of polarized macrophages (IV)

After 1-day macrophage polarization, complete medium containing the polarizing cytokines was removed, and M0, M1, and M2 macrophages were subjected to titanium particles suspended in complete medium at a concentration of $6x10^6$ particles/ml equaling about 10 particles / macrophage. Corresponding control macrophages received the particle-free complete medium. After 4 or 24 hours of particle stimulation, cell-culture supernatants were collected, centrifuged, and stored at -75 °C until used. Immediately after supernatant removal, cells were rinsed with PBS and total RNA was extracted with the RNeasy Mini Kit (Qiagen). Total RNA was stored at -75 °C until used; 4-hour total

RNA samples were used for microarray and quantitative RT-PCR analyses and 24-hour cell-culture supernatants for protein suspension array.

7.5.5. Live-cell and time-lapse imaging (IV)

Macrophage cultures maintained in 24-well plates as described were examined under and photographed with an Eclipse Ti-E inverted microscope (Nikon Instruments, Melville, NY, USA) in phase-contrast mode, with an environmental chamber capable of maintaining standard cell culture conditions (5% CO_2-in-air at +37°C), Prior ProScan III motorized XY stage and DS-QiMc camera with an NIS-Elements advanced research software version 3.1. ensemble (all components and software from Nikon). Monocyte-to-macrophage differentiation and subsequent macrophage polarization was documented by images taken once a day.

Macrophage-particle co-cultures were observed with time-lapse microscopy starting from 15 min after addition of the particles. Images of the co-cultures were taken at 15-min intervals; from pre-fixed, representative locations, for 6 hours, and several additional images were taken 24 hours after addition of the particles from representative locations.

Phagocytotic activity of the different macrophage types was assessed by quantifying the particles remaining outside the cell at a given time point in a total of six randomly chosen view fields per cell type. To this end, an automated image analysis algorithm was developed to measure the particles left in the background (=outside the cells) of each image. Briefly, the background was first segmented from cells and particles based on its color (most frequent color ± 5 grayshades in a 8-bit image) in Matlab (The MathWorks, Natick, MA, USA). Particles were segmented from cells based on their size (1-50 pix) and circularity (>0.85) by ImageJ.

Motility of the different macrophage types was assessed from 6-hour follow-up images by use of a manual tracking plug-in for ImageJ (v. 1.43u National Institute of Health, Bethesda, MD, USA). Briefly, the velocity of 120 randomly chosen cells, in six view fields per cell type, was determined by manually tracking the approximated focal point of the cell.

7.6. Microarray (IV)

Genome-wide expression profiling and subsequent data-analysis of titanium particle-induced trascriptome changes in different macrophage types was performed in the Biomedicum Functional Genomics Unit (Helsinki, Finland). Four-hour particle-stimulated and corresponding control macrophages of one representative donor were included in the analysis. This experiment was performed following protocols provided by the microchip manufacturer (Illumina, San Diego, CA, USA). The quality of the extracted total RNA was evaluated by NanoDrop 1000 Spectrophotometer (Thermo Scientific, Wilmington, DE, USA) and Agilent Bioanalyzer 2100 system (Agilent Technologies, Santa Clara, CA, USA). Total RNA was then used for complementary RNA (cRNA) in vitro transcription and labeling by the TotalPrep RNA Amplification Kit (Illumina). A normalized amount of labeled cRNA was hybridized with the probes on HumanHT-12 v4 Expression BeadChip in anhybridization oven, followed by washing and staining with Cy3-streptavidin (all from Illumina). The BeadChip was scanned with iScan with iScan Control Software, and the data were analyzed with GenomeStudio (2010.2) with the Gene Expression Module (1.7.0, all from Illumina).

Genes with a fold difference in expression ≥1.5 between particle-stimulated and unstimulated macrophages were considered significant and included in additional analysis.

Gene ontology enrichment analysis was by Fisher's Exact Test, and involved comparing the frequency of each present GO term to its frequency in a reference gene set. Multiple hypotheses-corrected *P*-values of less than 0.05 were considered significant. Signaling pathway impact analysis (SPIA) performed as described by Tarca et al (2009).

7.7. cDNA synthesis and qRT-PCR (III, IV)

The amount and quality of the total RNA extracted from 4-hour particle-stimulated- and unstimulated human M0, M1, and M2 macrophages (Study IV); 0-, 2-, 6-, 10-, and 24-h particle-stimulated and unstimulated mouse RAW 264.7 macrophages (Study III); and frozen synovial and interface tissues (in the TLR-expression profiling study), was measured with a NanoDrop 1000 spectrophotometer (Thermo scientific). Complementary DNA (cDNA) was then synthesized, using an equal amount of total RNA from each sample and either a VILO Superscript cDNA synthesis kit (Invitrogen, Carlsbad, CA, USA) in Study III or iScript cDNA Synthesis Kit (Bio-Rad, Hercules, CA, USA) in Study IV and in the TLR-expression profiling study.

For quantitative real-time PCR,we prepared a reaction mix comprising sample cDNA, a pair of purpose-designed human or mouse forward and reverse primers (Tables 5 and 6), and iQ SYBR Green Supermix (Bio-Rad). qRT-PCR was then done with iQ5 Real-Time PCR Detection System (Bio-Rad). In Study III, results were normalized to β-actin (ACTB), whereas three housekeeping genes: ACTB, porphobilinogen deaminase (PBGD), and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) served for normalization of the results in Study IV. In the TLR expression profiling study, two housekeeping genes, PBGD and large ribosomal protein P0 (RPLP0), served for results normalization.

In Study III, TLR quantification was done by comparing the fluorescence of sample PCR products with the fluorescence of a dilution series of an external standard of each TLR in question. In Study IV, and in the TLR expression profiling study, results were obtained by use of the comparative Ct method (Schmittgen and Livak 2008).

Primer pairs were designed with Beacon designer software (v7.5 Premier Biosoft international, Palo Alto, CA, USA) and the National Center for Biotechnology Information BLAST online program. For Study III primers were manufactured by Proligo (Paris, France) and for Study IV and for TLR expression profiling study by Oligomer (Helsinki, Finland).

Table 5. Human primer sequences used in Study IV and in the TLR expression profiling study. bp – Base pairs; OSM - oncostatin M; IL - interleukin; CCL - chemokine (C-C motif) ligand; CXCL - chemokine (C-X-C motif) ligand; TNFSF - tumor necrosis factor super family; RANKL - receptor activator of nuclear factor kappa B ligand; OPG - osteoprotegerin; TRAP - tartrate-resistant acidic phosphatase; CTSK - cathepsin k; TLR - toll-like receptor; ACTB - β-actin; GAPDH - glyceraldehyde 3-phosphate dehydrogenase; PBGD - porphobilinogen deaminase; RPLP0 - large ribosomal protein P0.

Gene	Primer sequence	Product (bp)
OSM	Forward (5') GGGAGGCGCTGCTCTAAGTCG Reverse (3') GGACGCTGCTCAGTCTGGTCCTTG	331
IL-6	Forward (5') AGGAGACTTGCCTGGTGAAA Reverse (3') GAGGTGCCCATGCTACATTT	329

IL-8	Forward (5') ACTTCTCCACAACCCTCTGG Reverse (3') TCTGCAGCTCTGTGTGAAGG	229
CCL2	Forward (5') ATTCCCCAAGGGCTCGCTCA Reverse (3') GGTTTGCTTGTCCAGGTGGTCC	231
CCL3	Forward (5') GGCTTCGCTTGGTTAGGAAGATGA Reverse (3') CAGAAGGACACGGGCAGCAGAC	294
CCL4	Forward (5') GAGCAGCTCAGTTCAGTTCCAGGTC Reverse (3') AGTAGCTGCCTTCTGCTCTCCAGCG	248
CCL20	Forward (5') ACTCCACCTCTGCGGCGAAT Reverse (3') CTGCCGTGTGAAGCCCACAA	106
CXCL2	Forward (5') TAAGGGCAGGGCCTCCTTCAGG Reverse (3') TGGGCAGAAAGCTTGTCTCAACCCC	141
TNFSF9	Forward (5') CAGCAGAACATTTTGGGCCACCA Reverse (3') AGCTTTCGCCCGACGATCCC	83
TNFSF14	Forward (5') TGCTGGGTTGACCTCGTGAGAC Reverse (3') CGGGTGGGTCTGGGTCTCTTGC	177
RANKL	Forward (5') GAGCGCAGATGGATCCTAAT Reverse (3') GCTTCAAGCTTGCTCCTCTT	274
OPG	Forward (5') TGTGAGGAGGCATTCTTCAG Reverse (3') GGTTAGCATGTCCAATGTGC	265
TRAP	Forward (5') CTGTCCTGGCTCAAGAAACA Reverse (3') CCATAGTGGAAGCGCAGATA	299
CTSK	Forward (5') ACCCAACAGGCAAGGCAGCTAA Reverse (3') GCAATGCCACAGGCGTTGTTCT	325
TLR1	Forward (5') CGGAGGCAATGCTGCTGTTCAG Reverse (3') TGTAGGGGTGCCCAATATGCCT	130
TLR2	Forward (5') GCTGCTCGGCGTTCTCTCAGG Reverse (3') TGTCCAGTGCTTCAACCCACAACT	190
TLR3	Forward (5') TGCCGTCTATTTGCCACACACTTC Reverse (3') GTGCACTTGGTGGTGGAGGATGC	175
TLR4	Forward (5') CCTGCGTGGAGGTGGTTCCTA Reverse (3') CCAGAAAAGGCTCCCAGGGCTA	289
TLR5	Forward (5') TGTTGGCGCTGTCCGAACCT Reverse (3') AGGTGGTCTCCCATGATCCTCG	211
TLR6	Forward (5') AAGAGATCTTGAATTTGGACTCATATC Reverse (3') TGAAGCTCAGCGATGTAGTTC	278
TLR7	Forward (5') TCTTGGCACCTCTCATGCTCTGC Reverse (3') GTGAGGTTCGTGGTGTTCGTGGG	281
TLR8	Forward (5') CTGCGCTGCTGCAAGTTACGGA Reverse (3') TTGCCCACCGTTTGGGGAACTTC	234
TLR9	Forward (5') CCCAGCATGGGTTTCTGC Reverse (3') ACTTCAGGAACAGCCAGTTG	160
ACTB	Forward (5') CAGCGGAACCGCTCATTGCCAATGG Reverse (3') TCACCCACACTGTGCCCATCTACGA	295
GAPDH	Forward (5') AAGGTCATCCCTGAGCTG Reverse (3') TGCTGTAGCCAAATTCGTTG	310
PBGD	Forward (5') AGATGCGGGAACTTTCTCTG Reverse (3') ACATGCCCTGGAGAAGAATG	237
RPLP0	Forward (5') GGCGACCTGGAAGTCCAACT Reverse (3') CCATCAGCACCACAGCCTTC	149

Table 6. Mouse primer sequences used in Study III. bp – Base pairs; TLR - toll-like receptor; ACTB - β-actin.

Gene	Primer sequence	Product (bp)
TLR1	Forward (5') GCTGGCCTGACTCTTACAGG Reverse (3') TCTGGATGAAGTGGGGAGAC	465
TLR2	Forward (5') GACTCACAGCAGCCATGAAA Reverse (3') TCGCGGATCGACTTTAGACT	451
TLR4	Forward (5') GCTTTCACCTCTGCCTTCAC Reverse (3') AGGCCCCAGAGTTTTGTTCT	432
TLR5	Forward (5') ATTCCTCGTCATCACCCTTG Reverse (3') TGCTTTTGCAGAAACCCTCT	480
TLR8	Forward (5') CTTTCCAGCACTTCCCTCAG Reverse (3') GAAGACGATTTCGCCAAGAG	460
TLR9	Forward (5') TGTCCTTCCTACCCAACCTG Reverse (3') AAGAGTGAAAGGCCAAAGCA	454
ACTB	Forward (5') CTTCTTTGCAGCTCCTTCGT Reverse (3') GTGCCAGATCTTCTCCATGT	310

7.8. Protein suspension array (IV)

Levels of 39 chemokines, cytokines, and growth factors in 24-hour particle-stimulated and corresponding control M0, M1, and M2 macrophage culture supernatants were measured with the Milliplex MAP Human Cytokine/Chemokine premixed 39 Plex kit (Millipore, Billerica, MA, USA) and Bio-Plex Suspension Array System (Bio-Rad Laboratories). A pooled cell culture supernatant sample was prepared by combining equal volumes of culture supernatant derived from four independent particle stimulation experiments. This pooled sample was analyzed in two technical replicates. A 20% increase or decrease in cytokine-, chemokine-, or growth-factor concentration compared to that in control cells was considered significant. Cytokines included in the analysis were CCL2, CCL3, CCL4, CCL7, CCL11, CCL22, chemokine (C-X3-C motif) ligand (CX3CL) 3, CXCL1, CXCL10, granulocyte colony-stimulating factor (G-CSF), GM-CSF, VEGF, epidermal growth factor (EGF), TGF-α, fibroblast growth factor (FGF)-2, fms-like tyrosine kinase 3 ligand (FLT-3 ligand), IFN-α2, IFN-γ, IL-1α, IL-1β, IL-1ra, IL-2, sIL-2rα, IL-3, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-12 (p40), IL-12 (p70), IL-13, IL-15, IL-17, sCD40L, TNFα, and TNFβ.

7.9. Statistical analyses (I, III, IV)

Statistical analyses used R (version 2.9.0, Studies I, III) or Graphpad Prism version 4.0 (GraphPad Software, La Jolla, CA, USA, Study IV and the TLR-expression profiling study). Results are expressed as mean ± standard error of mean and two-sided *P* values less than 0.05 were considered significant. A nonparametric Mann-Whitney test served for pair-wise comparison of variables in Studies I, III, and in the TLR-expression profiling study. A nonparametric Friedman test with Dunn's post-hoc test for matched groups allowed comparison of differences between groups in Study IV.

8. Results

8.1. Histopathology of aseptic and septic THR loosening (I, II)

8.1.1. Tissue architecture and cell populations

In control synovial samples, well-vascularized and, in some areas, slightly edemic loose connective tissue stroma was visible under a typical synovial lining layer. The tissue stroma was relatively acellular, and the most common cell type was $HSP47^+$ fibroblast, whereas inflammatory cell populations were scarce. Some scattered $CD68^+$ and $CD163^+$ macrophages were, however, evident and represented the second most common cell population encountered. Macrophages observed were small and did not form larger clusters. Minimal infiltrations by scattered $CD3^+$T cells and by small groups of $CD20^+$ B cells also occurred, whereas no $CD138^+$ plasma cells were visible. Neutrophils dectected were intravascular.

In aseptic synovial membrane-like interface tissues, two primary types of tissue architecture appeared: in some samples, large areas of relatively acellular and collagen-rich fibrotic tissue were visible, whereas in other samples an intensive and cell-rich foreign body reaction was observable. In fibrotic areas, the $HSP47^+$ fibroblast was the most commonly encountered cell, and in the areas of foreign-body reaction, loose connective tissue stroma was heavily infiltrated by large $CD68^+$ and $CD163^+$ macrophages forming sheet-like infiltrates, foreign body giant cells and some granulomas. By polarized light microscopy, phagocytosed UHMWPE particles were detectable in most samples and, in samples with apparent metallosis, phagocytosed metal particles were also visible. Some scattered $CD3^+$ T cells and $HSP47^+$ fibroblasts were located between the large macrophage infiltrates. No apparent neutrophil, $CD20^+$ B cell, or $CD138^+$ plasma cell infiltration into tissue was detectable.

In septic synovial membrane-like interface tissues, some samples or sections showed large areas of necrosis and fibrosis, whereas other samples or sections consisted of loose connective tissue heavily infiltrated by heterogeneous inflammatory cell populations interspersed among $HSP47^+$ fibroblasts. Large $CD68^+$ and $CD163^+$ macrophage infiltrates consisting of large macrophages organized in sheet-like infiltrates and foreign body giant cells were visible. Some phagocytosed wear particles were detectable. Neutrophils formed considerable infiltrates in some, but not all, areas. High numbers of scattered and nodular CD3-positive T lymphocytes and also nodular $CD20^+$ B cell infiltrates were present. Some of the B cells had been activated to the $CD138^+$ plasma cell.

Summary of the cell populations present in control, aseptic, and septic interface tissues is in Figures 4 and 6.

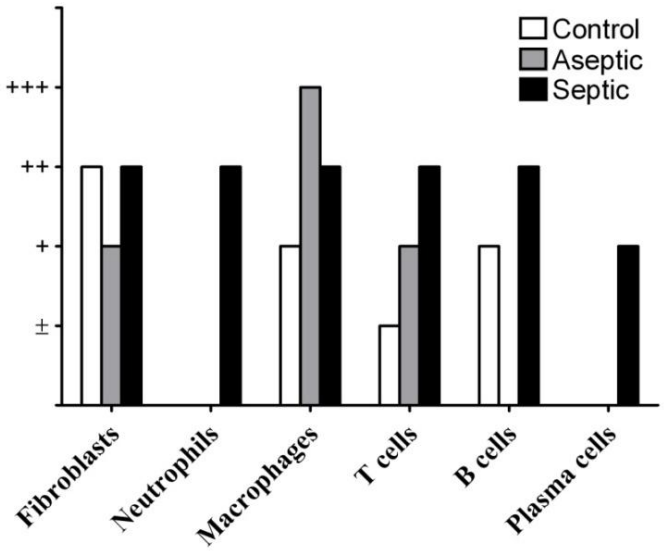

Figure 4. Cell populations in osteoarthritic control synovial membrane, aseptic, and septic interface tissues were evaluated by immunohistochemistry with cell-type-specific antibodies (Study II). The number of positive cells in each tissue was evaluated on a semiquantitative scale (vertical axis), with no positive staining (-); occasional positive cells (±); some positive cells (+); moderate numbers of positive cells (++); and large numbers of positive cells (+++).

8.1.2. TLR expression and cell localization

In control synovial samples, all TLRs were present, and the mean number of TLR^+cells was 26.9 ± 8.3 per high-power field (Table 7). In control samples, TLR immunoreactivity occurred mainly in the vascular endothelium and in some synovial lining cells. Occasional macrophages and fibroblasts in tissue stroma were also positive for the TLRs. No clear difference in staining pattern or cell populations of different TLRs was detectable, although the number of cells positive for TLR9 appeared to be markedly low. In qRT-PCR analysis, all of the TLRs were expressed in the control synovial tissues (Table 8, Pajarinen J, Jämsen E, Konttinen YT, unpublished results).

In aseptic synovial membrane-like interface tissues, all TLRs were present, with the mean number of TLR-positive cells being 56.9 ± 14.7 per high-power field (Table 7). In addition to vascular endothelium, fibroblasts, and occasional synovial lining cells, sheet-like macrophage infiltrates, and foreign body giant cells were heavily positive for all of the TLRs. No clear difference occurred in the staining pattern among TLRs, because the infiltrating macrophages seemed to be positive for all of the TLRs. In qRT-PCR analysis, all of the TLRs were expressed in the aseptic interface tissue, and the expression of all TLRs except TLR3 and TLR7 was significantly higher than in control tissues. Similarly, in comparison to control tissues, the expression of the macrophage-derived chemokines IL-8, CCL2, CCL3, and CCL4, as well as osteoclast markers TRAP and cathepsin K were significantly upregulated and OPG downregulated, while RANKL was unaffected (Table 8).

In septic synovial membrane-like interface tissues, TLR immunoreactivity appeared especially in macrophages but also in infiltrating neutrophils and in vascular endothelium. Infiltrating lymphocytes were TLR positive, but the exact identity of these cells could not be confirmed. No clear difference in staining pattern among TLRs was detectable because

the infiltrating inflammatory cell populations, mainly macrophages and neutrophils, seemed to be positive for both of the TLRs.

Summary of the TLR localization in control, aseptic, and septic interface tissues appears in Figures 5 and 6.

8.1.3. Control stainings

All control immunohistochemical stainings done with irrelevant IgGs used at the same concentration but instead of the primary antibodies were negative.

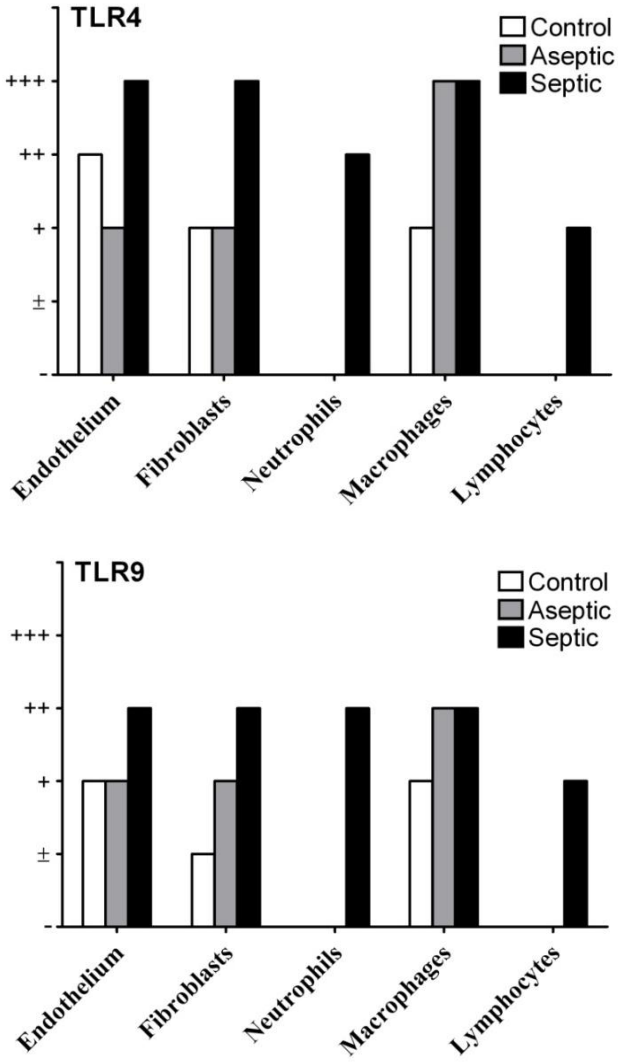

Figure 5. TLR4 and TLR9 localization in various cell types in osteoarthritic control synovial membrane, aseptic, and septic interface tissues as evaluated by immunohistochemistry (Study II). Vertical axis as in Figure 4.

Table 7. Mean number of TLR immunoreactive cells in controlosteoarthritic synovial membrane and in aseptic interface tissues as determined by immunohistochemical staining (Study I). TLR - toll-like receptor.

Receptor	Control	Aseptic
TLR1	22 ± 4.1	49 ±14.2
TRL2	27.8 ± 2.7	61 ± 22.3
TLR3	36.8 ± 10.1	75.3 ± 19.5
TLR4	22.3 ± 5.4	71.8 ± 26.2
TLR5	24.5 ± 4.6	45.8 ± 18.9
TLR6	14.8 ± 1.4	54 ± 15
TLR7	26.8 ± 8.6	56.5 ± 14.5
TLR8	40.5 ± 7.3	69 ± 17.1
TLR9	3.8 ± 2.8	40.5 ± 9.0

Table 8. Expression of selected chemokines (A), osteoclast markers (B), and TLRs (C) in control osteoarthritic synovial tissues and in aseptic interface tissue as determined by qRT-PCR (Pajarinen J, Jämsen E, Konttinen YT, unpublished results). Fc - fold change; other abbreviations as in Table 5.

	Gene	Relative expression control	Relative expression aseptic	Fc	*P* value
A	***IL-8***	81 ± 32	1008 ± 319	12.4	0.001
	CCL2	1161 ± 250	4494 ± 930	3.9	0.015
	CCL3	5 ± 2	146 ± 62	29.7	<0.001
	CCL4	155 ± 50	696 ± 322	4.5	0.034
B	***RANKL***	6 ± 2	5 ± 2	-	ns
	OPG	245 ± 70	38 ± 15	-6.7	0.005
	TRAP	20 ± 5	1912 ± 313	95.6	<0.001
	CTSK	3213 ± 725	14777 ± 1806	4.6	<0.001
C	***TLR1***	59 ± 5	165 ± 20	2.9	<0.001
	TLR2	108 ± 24	196 ± 25	1.8	0.023
	TLR6	31 ± 6	63 ± 10	2	0.005
	TLR4	194 ± 42	279 ± 34	1.4	0.022
	TLR5	91 ± 15	166 ± 27	1.8	0.038
	TLR3	279 ± 56	190 ± 27	-	ns
	TLR7	399 ± 64	541 ± 103	-	ns
	TLR8	166 ± 21	438 ± 63	2.6	0.001
	TLR9	2.5 ± 0.5	4.7 ± 0.7	1.9	0.030

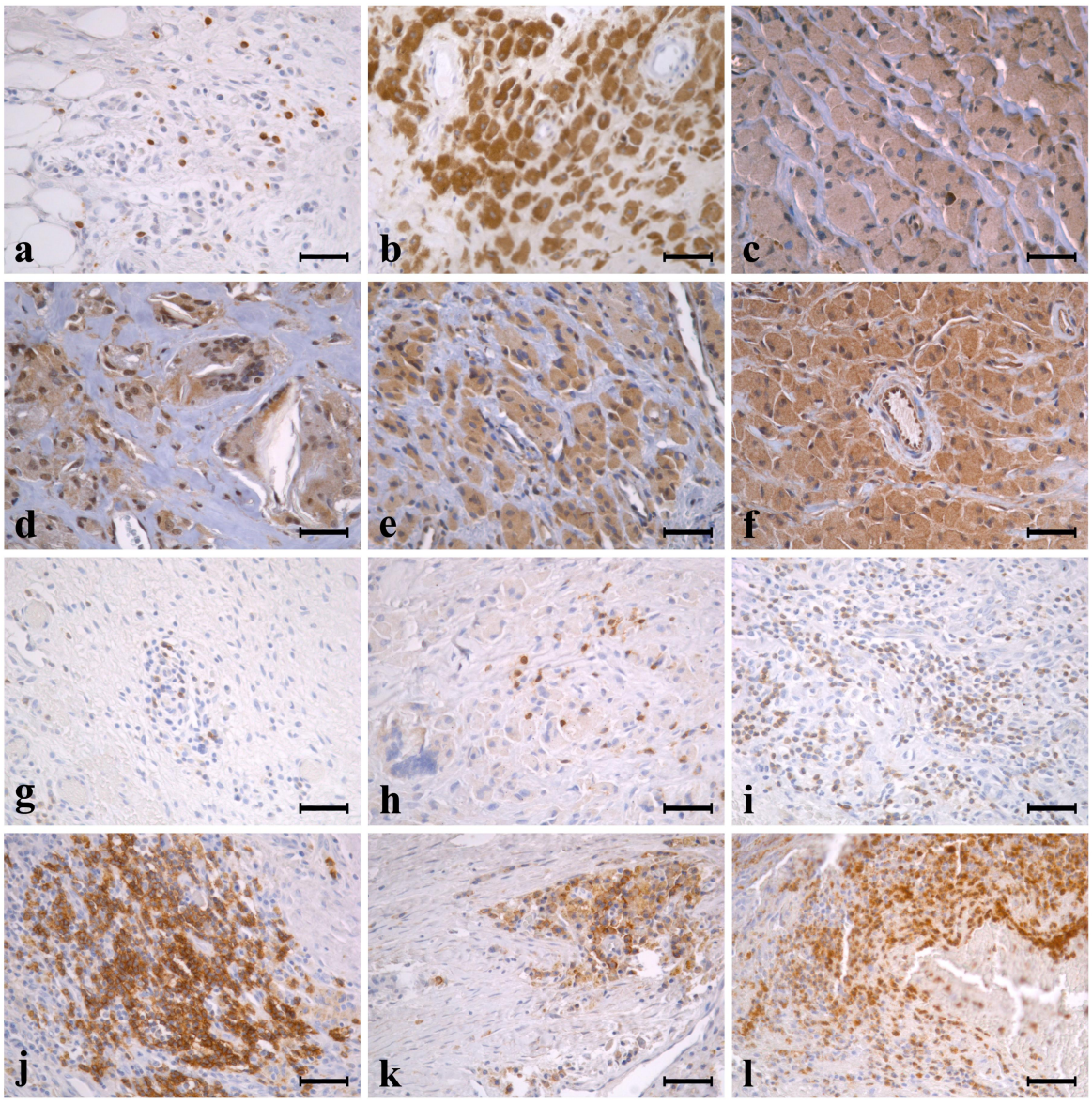

Figure 6. Immunohistochemical staining results of Studies I and II. In osteoarthritic synovial membrane, occasional small $CD68^+$ macrophages are visible, scattered among fibroblasts (a). In contrast, interface tissue from aseptic loosening is characterized by massive infiltration of $CD68^+$ macrophages and formation of foreign body giant cells (b). These large macrophages and foreign body giant cells express all of the TLRs investigated, including TLR1 (c), TLR2 (d), TLR4 (e) and TLR6 (f). In osteoarthritic synovial membrane (g) and in aseptic interface tissue (h) only occasional $CD3^+$ T lymphocytes are visible, scattered among the fibroblasts and macrophage infiltrates, but no B lymphocytes or plasma cells were detectable. In contrast, in septic loosening, diffuse infiltrates of $CD3^+$ T lymphocytes (j) and nodular infiltrates of $CD20^+$ B lymphocytes (j) and $CD138^+$ plasma cells (k) were observable. In septic interface tissue samples NE^+ neutrophil infiltrates were also detectable in some areas (l). Scale bars 50 µm. CD - cluster of differentiation; TLR - toll-like receptor; NE - neutrophil elastase.

8.2. Effect of wear particles on TLR expression (III)

8.2.1. Mouse model of particle-induced inflammation

In femur samples collected two weeks after the implantation, active bone remodeling and callus formation occurred in both study groups (stainless-steel rod with or without titanium particles). Inflammatory cells were visible in bone marrow. After ten weeks of implantation, inflammation and active bone remodeling had ceased, and callus had consolidated into compact bone. The rod channel was surrounded by circumferential woven bone that often formed struts radiating toward the cortical bone. In the particle group, titanium particles occurred in the intramedullary space and within the fibrous interface tissues. Some of the particles had been phagocytosed by bone marrow macrophage-like cells. TLR immunoreactive cells were visible among the bone-marrow, bone, endosteal, and periosteal cells. The number of TLR immunoreactive cells was generally higher in two-week samples than in ten-week samples, and additionally, the number of TLR-immunoreactive cells seemed to be reduced in the particle group at both time-points as compared to the non-particle group. A summary of the immunohistochemical staining results is in Figures 7 and 8.

8.2.2. In vitro mouse macrophage culture

Mouse monocyte/macrophage cell line RAW 264.7 expressed the investigated TLRs at the mRNA level, as indicated by qRT-PCR. Although TLR expression levels tended to be lower in the particle-stimulated cells than in non-stimulated control cells, no clear differences in TLR expression levels were detectable at any of the time-points during 24-hour particle stimulation.

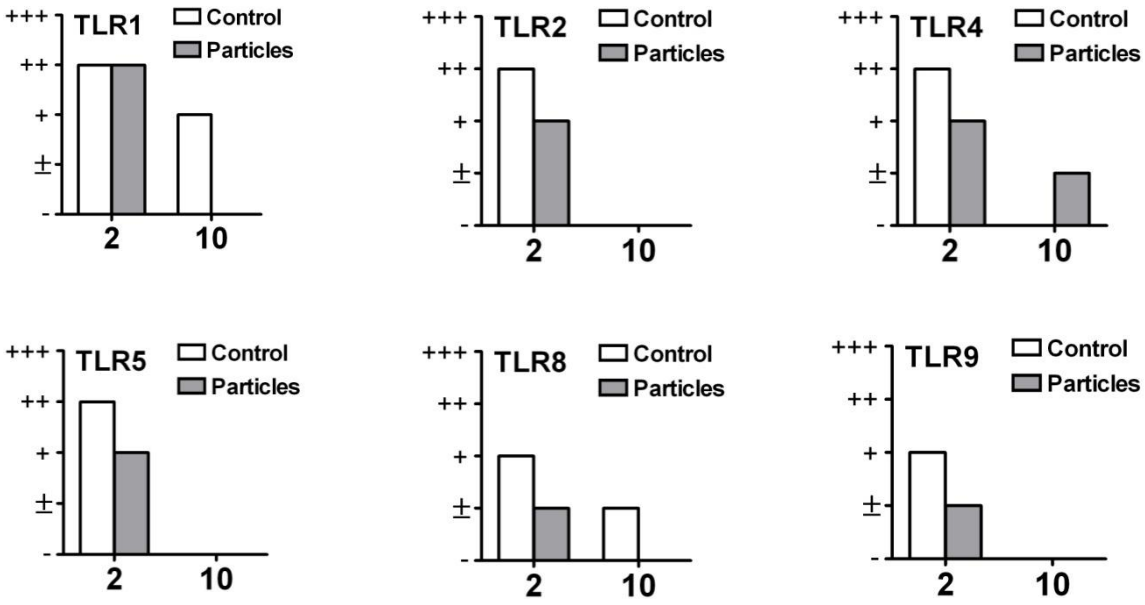

Figure 7. Number of TLR immunoreactive cells in endosteum, bone, and periosteum in a mouse intramedullary model of particle-induced inflammation (Study III). A stainless steel wire with (particles) or without (control) titanium particles was inserted bilaterally into the mouse femoral canal; the number of peri-implant TLR immunoreactive cells was evaluated two and ten weeks post-operatively. Vertical axis as in Figure 4.

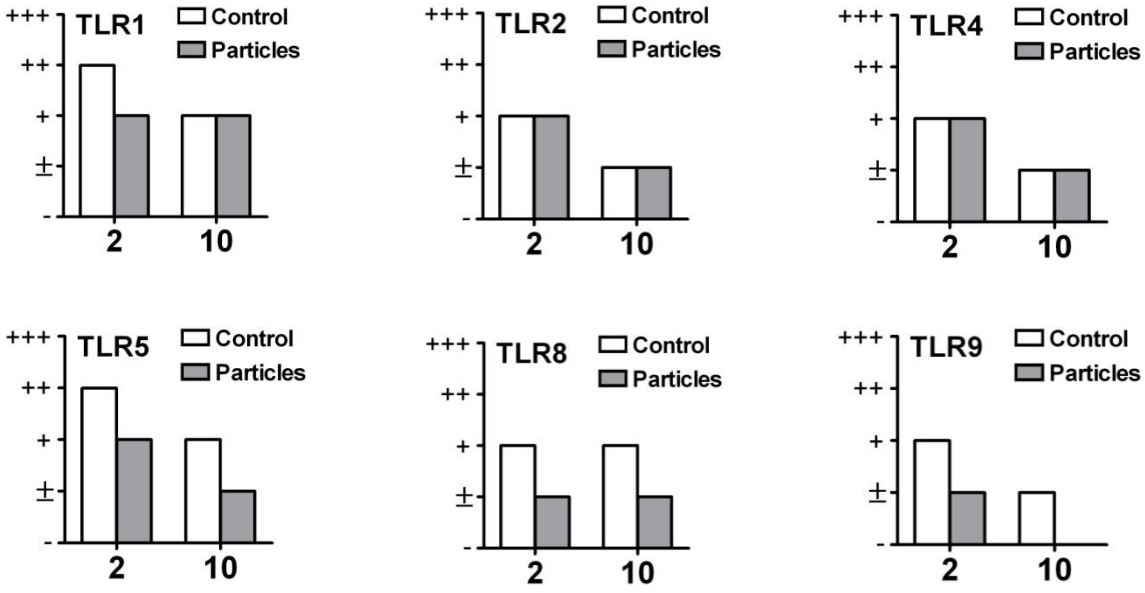

Figure 8. Number of TLR immunoreactive cells in bone marrow in a mouse intramedullary model of particle-induced inflammation (Study III). A stainless steel wire with (particles) or without (control) particles was inserted bilaterally into the mouse femoral canal, and the number of peri-implant TLR immunoreactive cells was evaluated two and ten weeks post-operatively. Vertical axis as in Figure 4.

8.3. Effect of macrophage polarization on wear-particle responses (IV)

8.3.1. Cell morphology, motility, and particle phagocytosis

Monocyte-to-macrophage differentiation and subsequent macrophage polarization had an impact on the cell morphology, motility, and phagocytotic activity of macrophage subtypes. During monocyte-to-macrophage differentiation, initially small and rounded monocytes adhered, spread to the culture surface, and actively proliferated, already by day one forming large colonies and spreading over the culture dish. After full macrophage differentiation and polarization, M0 macrophages were mostly elongated and spindle-shaped, M1 macrophages clearly rounded, and M2 macrophages mostly cone-shaped, likely reflecting their high mobility rate. After titanium-particle addition, rapid phagocytosis of particles was observable; only 15 min after particle addition, considerable numbers of particles appeared in the intracellular compartment of all three macrophage subtypes.

In time-lapse microscopy, M0 and M2 macrophages actively moved around the culture dish, at the same time phagocytosing foreign particles on a relatively large surface area, while M1 macrophages were practically stationary and phagocytosed titanium particles only from their immediate surroundings, leaving large quantities of titanium particles between their cell bodies. During a 6-hour follow-up, the average speed of M0 macrophages was 5.0 nm/s ± 1.4, of M1 macrophages 2.6nm/s ± 0.65, and of M2 macrophages 6.3 nm/s ± 1.5. After 24 hours of particle addition and phagocytosis, the percentage of background area covered by particles, and thus not phagocytosed by macrophages, was 0.43% ± 0.01 for M0 macrophages, 0.86 ± 0.09 for M1 macrophages, and 0.43% ± 0.02 for M2 macrophages.

8.3.2. Microarray and qRT-PCR

A summary of 4-hour titanium particle stimulation-induced transcriptome changes in M0, M1, and M2 macrophages is in Table 9 and a selected list of these particle-induced genes in various macrophage types with fold change ≥ 2 in Table 10 (with a more detailed listing of the genes involved provided in the original publication). The most prominent wear particle responses occurred in M1 macrophages and the least in M2 macrophages. The wear particle-induced transcriptome changes were highly unique in each macrophage subtype, with only one gene in common for the wear particle response of all three cell types, RND3, up-regulated in all three macrophage subtypes. Eight genes were up-regulated both in M0 and M1 macrophages (IL-8, CCL20, JUN, IRAK2, DUSP5, GEM, HES4, ZC3H12C), whereas only one gene was in common in the wear-particle response of M0 and M2 macrophages (MARCKSL1). Three genes were up-regulated in both M1 and M2 cells (ATF4, IER3, HEG1), whereas one gene was up-regulated in M1 macrophages but down-regulated in M2 macrophages (CXCL1).

GO and SPIA analyses were subsequently performed to identify possible functional groups of genes or signaling pathways that might be activated by wear-particle stimulation in the three macrophage subtypes. A summary of these analyses is in Table 11 (with a more detailed listing of the GO terms involved provided in the original publication).

To verify results of the microarray experiment, qRT-PCR was performed for selected genes in a larger sample size (Table 12).

Table 9. Summary of the effect of macrophage polarization on wear-particle-induced transcriptome changes (Study IV). M0, M1, and M2 macrophages were subjected to titanium particles for four hours, and the subsequent transcriptome changes in each macrophage subtype analyzed by genome-wide microarray analysis. Number of genes involved and up- or down -regulated in each macrophage subtype is included . "Unique" signifies the percentage of genes up- or down-regulated only in the corresponding macrophage subtype.

Cell type	Tota no. of genes altered	Up-regulated genes	Down-regulated genes	Unique
M0	63	32	31	84%
M1	192	145	47	94%
M2	59	33	26	92%

Table 10. Selected particle-induced or suppressed genes among macrophage types (Study IV). M0, M1, and M2 macrophages were subjected to titanium particles for four hours, and the subsequent transctiptome changes in each macrophage subtype analyzed by genome-wide microarray analysis. Only genes with fold change of ≥ 2 are presented. In M0 macrophages, no genes with fold change of ≥ 2 were detectable.

M0		
Gene	**Description**	**Fold change**
-	-	-
M1		
Gene	**Description**	**Fold change**
CCL3L1	chemokine (C-C motif) ligand 3-like 1	4.6
CCL3	chemokine (C-C motif) ligand 3	4.2
CCL20	chemokine (C-C motif) ligand 20	4.1
CCL4L2	chemokine (C-C motif) ligand 4-like 2	3.2
TNFSF9	tumor necrosis factor (ligand) superfamily, member 9	3.2
OSM	oncostatin M	2.6
IL-1β	interleukin 1, beta	2.4
CXCL2	chemokine (C-X-C motif) ligand 2	2.3
IL-8	interleukin 8	2.2
PTGS2	prostaglandin-endoperoxide synthase 2	2.2
M2		
Gene	**Description**	**Fold change**
PLXND1	plexin D1	0.5
MARCKSL1	MARCKS-like 1	0.5
FMNL1	formin-like 1	0.5
THBS1	thrombospondin 1	0.5
CABP5	calcium- binding protein 5	0.5

Table 11. Results of the gene ontology (GO) and signaling pathway impact analyses (SPIA) of the microarray data (Study IV). M0, M1, and M2 macrophages were subjected to titanium particles for four hours, and the subsequent transcriptome changes in each macrophage subtype analyzed by genome-wide microarray analysis (Study IV). To identify possible functional groups or signaling pathways activated, GO (A) and SPIA (B) analyses of microarray expression data were necessary. (A) In M0 macrophages, one GO term was significantly enriched, in M1 macrophages, 115 different GO terms were significantly enriched, but none in M2 cells. The ten statistically most enriched GO biological process terms in M1 macrophages are shown. (B) According to the SPIA, two signaling pathways were activated in M0 macrophages, four in M1 macrophages, and none that could be recognized in M2 macrophages.

A	**Cell type**	**GO term**	**Number of Genes**	***P* value**
	M0	Chemokine activity	4	0.029
	M1	Inflammatory response	26	<0.001
		Immune response	35	<0.001
		Response to external stimulus	32	<0.001
		Chemotaxis	16	<0.001
		Negative regulation of cellular process	55	<0.001
		Regulation of phosphorylation	26	<0.001
		Negative regulation of kinase activity	12	<0.001
		Signal transduction	61	<0.001
		Cell communication	51	<0.001
		Apoptosis	36	<0.001
	M2	-	-	-
B	Cell type	**SPIA pathway**	**Number of genes**	***P* value**
	M0	Chemokine signaling	6	0.0054
		Toll-like receptor signaling	5	0.0132
	M1	Cytokine-receptor interaction	19	<0.001
		Chemokine signaling	13	<0.001
		NOD-like receptor signaling	9	<0.001
		Toll-like receptor signaling	7	<0.001
	M2	.	-	-

Table 12. Results of the qRT-PCR (Study IV). M0, M1, and M2 macropages were subjected to titanium particles for four hours, and the expression levels of selected genes were analyzed by qRT-PCR. Data represented as fold change between particle-stimulated and unstimulated samples. * = $P<0.05$ between M1 and M2. Abbreviations as in Table 5.

Gene	**M0**	**M1**	**M2**
OSM	3.4 ± 1.0	9.6 ± 2.1*	2.0 ± 0.4
IL-8	2.6 ± 0.3	4.7 ± 0.5	2.5 ± 0.4
CCL3	2.1 ± 0.2	4.7 ± 0.9*	1.4 ± 0.2
CCL20	3.9 ± 0.6	8.6 ± 4.0	2.3 ± 1.0
CXCL2	3.5 ± 1.0	5.9 ± 1.0	2.9 ± 1.2
TNFSF9	2.8 ± 1.0	6.7 ± 1.1*	1.1 ± 0.1
TNFSF14	1.9 ± 0.4	2.5 ± 0.3	1.2 ± 0.1
IL-6	1.7 ± 0.3	8.0 ± 4.2	1.1 ± 0.1

8.3.3. Protein suspension array

An overview of 24-hour titanium-particle stimulation-induced qualitative proteome changes in M0, M1, and M2 macrophages is in Table 13 (with more detailed listing of mediators similarly or differentially regulated provided in the original publication). Qualitatively, M0 and M1 macrophage wear-particle-response profiles resembled each other, while the M2 response profile was more distinct from the other two macrophage types. However, quantitative analysis of wear particle responses revealed that the wear-particle responses of M0 and M1 macrophages were, in fact, also dissimilar and, in comparison to M0 macrophages, the wear-particle responses in M1 macrophages were generally enhanced, as detailed in Table 14. In M2 macrophages, chemotactic and pro-inflammatory particle responses typical for M0 and M1 cells were effectively suppressed, although an increase in IL-8, CCL7, and IL-1α and a decrease in IL-10 production occurred (Table 14). Interestingly, wear-particle stimulation had no effect on production of IFN-α2, IFN-γ, or IL-4 in any macrophage subtype.

In addition to wear-particle responses, some observations about the nature of different macrophage subtypes were possible. Unstimulated M0 and M1 macrophages produced high basal levels of CXCL10, while M2 macrophages displayed high basal secretion of CCL22, IL-10, IL-1ra, and TGFα. In addition, M1 macrophages produced high amounts of IFN-γ, and M2 macrophages of IL-4, likely reflecting positive auto- and paracrine feedback loops.

Basal secretion levels of CCL2 were very high and beyond the suspension-array detection range in all of these macrophage subtypes. Likewise, the production of IL-8 and CXCL10 was over the arrays' detection range in particle-stimulated M0 and M1 macrophages. and of CCL22 in both stimulated and unstimulated M2 macrophages. No IL-2, IL-3, IL-5, IL-9, IL-13, IL-17, or TNF-β was produced by any of the macrophage types, neither at basal level nor after particle stimulation.

Table 13. Summary of the effect of macrophage polarization on wear-particle-induced proteome changes (Study IV). M0, M1, and M2 macropages were subjected to titanium particles for 24 hours, and subsequently the concentration of 39 cytokines, chemokines, and growth-factors in the cell culture media was evaluated by protein suspension array. The total number of mediators, as well as the number of mediators, increased or decreased by wear- particle stimulus in macrophage subtypes is presented. "Unique" indicates the number of mediators exclusively up-regulated/down-regulated in the corresponding macrophage subtype.

Cell type	Total	Increased	Decreased	Unique
M0	20	17	3	2/1
M1	22	22	0	5/0
M2	10	3	7	1/6

Table 14. Cytokine levels (pg/µl) in particle-stimulated (+) and unstimulated (-) macrophage subpopulations as determined by protein suspension array. M0, M1, and M2 macrophages were subjected to titanium particles for 24 hours, and subsequently the concentration of 39 cytokines, chemokines, and growth-factors in the cell culture media was evaluated by protein suspension array. In comparison to M0 macrophages, the wear-particle responses in M1 macrophages were generally enhanced, and especially the production of mediators listed under A was higher in M1 than in other macrophage types. Mediators in section B were produced in similar magnitudes by both particle-stimulated M0 and M1 macrophages. Particle responses in M2 macrophages were generally suppressed, although an increase in IL-8, CCL7, and IL-1α and a decrease in IL-10 production occurred (B, C, D). Type I interferon INF-α2 remained unaffected by particle stimulation. In addition to having an impact on macrophage wear-particle responses, basal secretion of several mediators was affected by macrophage polarization (D). (#) over assay's detection range. For abbreviations see section 7.8.

		M0		**M1**		**M2**	
		-	+	-	+	-	+
A	**TNFα**	9.5	54.5	54.5	323.8	18.5	18.5
	IL-1β	1.2	4.2	1.9	7.2	1.4	1.0
	CCL3	54.6	266.8	83.7	1282.9	74.1	67.6
	IL-7	8.1	10.6	10.6	23.1	9.4	8.8
	IL-12p40	5.0	4.6	5.3	11.0	7.9	5.7
	IL-12p70	1.8	2.1	2.7	3.9	1.6	1.4
	GM-CSF	5.2	11.1	7.5	23.6	7.8	7.9
	G-CSF	1.2	2.9	2.1	8.3	7.5	5.4
	EGF	12.5	11.1	6.3	13.5	11.5	10.9
	sIL-2RA	3.5	3.6	4.9	9.4	5.0	4.7
	sCD40L	16.6	11.9	13.7	18.9	7.1	7.1
	FLT3lig.	11.3	13.5	10.0	19.9	13.0	11.9
B	**IL-6**	6.8	9.4	7.0	9.4	8.0	5.2
	CCL4	411.7	1084.1	333.0	1253.1	748.7	693.1
	CCL7	408.3	620.3	568.9	768.2	186.8	232.2
	CCL11	20.3	25.5	20.2	32.2	15.6	16.8
	CXCL1	1119.3	1576.6	888.5	1235.0	300.8	281.3
	FGF2	19.6	28.2	21.1	32.8	20.6	19.8
	VEGF	80.9	141.4	96.3	184.8	67.9	74.9
C	**IL-8**	7124.0	#8946	7583.0	#9131	1372.0	2963,8
	IL-1α	24.1	21.6	27.8	27.6	2.8	6.3
	IFN-α2	29.3	31.0	31.2	35.9	22.4	22.4
D	**CXCL10**	#29121	#26495	#28671	#30053	1651.1	711.5
	CCL22	6467.0	4858.0	3223.0	4385	#41463	#29986
	IL-10	5.9	8.2	1.02	2.3	28.0	17.9
	IL-1ra	65.3	81.9	86.7	159.4	218.2	207.3
	TGFα	<1	<1	<1	<1	20.3	21.7
	IFNγ	22.9	24.5	639.1	613.7	6.0	4.5
	IL-4	194.9	177.4	193.4	208.6	3051.2	2927.2

9. Discussion

9.1. Histopathology of aseptic and septic THR loosening (I,II)

9.1.1. Cell populations of aseptic and septic interface tissue

We analyzed cell populations present in the aseptic and septic interface tissues and in control synovial tissue, using antibodies raised against cell-type-specific markers. We evaluated the presence and relative amount of tissue fibroblasts (HSP47), macrophages (CD68 and CD163), neutrophils (neutrophil elastase), T cells (CD3), B cells (CD20), and plasma cells (CD163).

In comparison to control synovial tissue that contained mostly fibroblasts and occasional macrophages, likely representing tissue-resident macrophages, the aseptic interface tissue was characterized by massive macrophage infiltrates composed of large $CD68^+$ and $CD163^+$ macrophages organized into sheet-like formations or chain-like structures. Foreign-body giant cell formation was also consistently observable. Some scattered T cells were apparent between macrophage infiltrates, but neutrophils, B cells, and plasma cells were absent. These findings are in agreement with various reports consistently describing the macrophages and foreign body giant cells as the hallmarks of the foreign body reaction in the aseptic interface tissues (Willert and Semlitsch 1977, Goldring et al 1983, Goodman et al 1989, Santavirta et al 1990, Jiranek et al 1993, Kim et al 1993, Boynton et al 1995, Goodman et al 1997, 1998). Likewise, the presence of some scattered T lymphocytes and the rarity or absence of neutrophils, B cells, and plasma cells is in line with previous findings (Jiranek et al 1993, Boynton et al 1995, Goodman et al 1997, Goodman et al 1998, Baldwin et al 2002, Arora et al 2003).

In septic synovial interface tissues, in addition to occasional areas of sheet-like macrophage infiltrates closely resembling those of the aseptic interface tissues and thus possibly reflecting underlying and developing foreign body reaction, all of the investigated inflammatory cell populations were detectable. In some areas, hundreds of neutrophils formed considerable infiltrates, thus fitting well into earlier descriptions defining the septic interface as containing 1 to 10 neutrophils in a high-power field. T and B lymphocytes as well as plasma cells also formed considerable infiltrates in some areas, with T cells displaying a more scattered distribution, and B cells and activated plasma cells forming clearly nodular structures.

Some uncertainty regarding these results stems from the somewhat untypical microbiological status of our sample set, namely the high proportion of *Propionibacterium acnes*-culturepositive tissue samples. *Propionibacterium acnes* is a common skin-derived contaminant in these cultures, and traditionally, *Propionibacterium* is thought to cause actual hip-replacement infections only rarely. This view is, however, somewhat challenged by recent reports describing *Propionibacterium* as a important and previously under-diagnosed agent causing hip replacement infections (Zeller et al 2007, Butler-Wu et al 2011, Perry et al 2011). The nature of *Propionibacterium acnes*-culture- positive findings in our sample set thus remains uncertain, but regardless of the evident possibility of false-positive results for bacterial cultures, no clear difference was detectable in the histological picture between infective agents.

The number of neutrophils in the interface tissue has served as a relatively useful histopathological marker of low-grade implant-related infection (Lonner et al 1996, Pace et al 1997, Della Valle et al 1999, Banit et al 2002, Francés Borrego et al 2006, Bori et al 2007, Nuñez et al 2007, Kanner et al 2008). Likewise, an increased number and

proportion of neutrophils in joint fluid aspirates has successfully served as a preoperative marker of implant-related infection (Spangehl et al 1999, Trampuz et al 2004, Ghanem et al 2008, Schinsky et al 2008, Cipriano et al 2012). The sensitivity and specificity of these methods have been extensively evaluated, whereas the usefulness of lymphocyte infiltrates in the histopathological diagnosis of implant-related infection has undergone only limited evaluation (Athanasou et al 1995, Pandey et al 1999). Similarly, reports evaluating the proportion of lymphocyte subpopulations in preoperative joint-fluid aspirates of suspected joint replacement infections are scarce (Niki et al 2006). This is a relevant lack of knowledge, as in theory, lymphocytes—especially B cells and plasma cells—would seem like ideal marker cells for implant-related infection; B cells and plasma cells are only rarely seen in aseptic interface tissues, likely do not participate in development of foreign-body reaction, and are classical, long-lived, markers of chronic inflammation. Thus, for detecting low-grade, implant-related infection that releases bacteria only periodically, such lymphocyte subpopulations might be better suited than are the neutrophils classically associated with acute, ongoing inflammation.

Aseptic loosening has traditionally been considered as primarily driven by innate immunity and by macrophages in particular. Conversely, the role of lymphocytes, and of the adaptive immunity response in general, in the pathogenesis of aseptic loosening is somewhat controversial and not well established. Activation of adaptive immunity requires that a specific antigen, processed and bound to MHC II, is presented to T cells in a local lymph node by matured dendritic cells that express sufficient numbers of co-stimulatory molecules. As it seems very unlikely that foreign-body particles could function as antigens that can stimulate T-cell activation, some other foreign-body reaction-related antigen should be mediating this process, assuming that T cell activation is assumed or observed. One possibility is that danger-signal molecules suspected to be concentrated on particle surfaces could function as such antigens. PAMPs would more likely be involved than alarmins, because self-molecules should generally not evoke any T-cell response. The route from the particles' surface-bound PAMP to MHC II still seems somewhat complex. Furthermore, if PAMPs were to effectively function as antigens and elicit a T-cell response, B cell activation would also be assumed. Based on this purely theoretical consideration, therefore, participation of the T cell in the foreign-body reaction seems unlikely.

Indeed, studies investigating the role of T cells in aseptic loosening generally have not found strong support for their role in its pathogenesis (Goodman 2007). For example, lymphocyte-derived marker cytokines, IL-2, IFNγ, or IL-4, are not uniformly found in the interface tissue; wear particles do not evoke T cell activation in co-cultures; and in mice models, the extent of osteolysis or observed foreign body reaction is independent of the existence of lymphocytes (Santavirta et al 1991, Goodman et al 1994, Jiranek et al 1995, Li et al 2001, Taki et al 2005, Rodriguez et al 2009). On the other hand, most studies investigating the cellular constituents of aseptic interface tissue have reported scattered T lymphocyte infiltrates, similar to ones observed in the current study, in the majority of cases. One estimate is that T cells can account for as much as 10% of the cellular constituents of the interface tissue (Baldwin et al 2002, Arora et al 2003). Another claim is that the majority of these are activated Th1 cells and that they support macrophage activation. These results are inconclusive, however, as other studies contradict them; the ultimate relevance of these T-cell infiltrates remains still to be determined (Goodman 2007). However, in the case of metal particles and ions released primarily from metal-on-metal implants, activated T cell infiltrates are more constantly seen and also are suspected

to play a larger role by participating in a cell-mediated hypersensitivity reaction against metal particles (Hallab et al 2001).

In contrast to T cells, B cells and plasma cells are detectable in only a minority of cases of aseptic loosening, and the role of plasma cells and antibodies in the pathogenesis of aseptic loosening is generally not considered significant (Goodman et al 1989, Santavirta et al 1990, Jiranek et al 1993, Kim et al 1993, Goodman et al 1998, Pandey et al 1999). Indeed, B cell maturation into plasma cells is a complex and multistep process that requires not only specific antigen recognition by the B cell receptor, but also a subsequent second activating signal from dendritic cell-activated T-helper cells. Thus the role of B cells in the pathogenesis of aseptic loosening seems even more unlikely than that of T cells.

As indicated by our results, however, B cells and plasma cells are clearly present in septic interface tissues, likely because living bacteria contain an abundance of potential lymphocyte-activating antigens. Once phagocytosed and disintegrated by antigen-presenting cells, these are sufficient to cause full-blown activation of the adaptive immune system, including activation of T cells and maturation of B cells into antibody-producing plasma cells. In this regard, it is tempting to speculate that the presence of $CD20^{+}$ B cells and mature plasma cells reported in some of the aseptic interface tissues might, in fact, indicate a subclinical biofilm-hidden infection that is mostly quiescent but occasionally releases bacteria. These bacteria cause intermittent activation of innate and adaptive immune systems which then clear the invading pathogen but also release osteolytic cytokines leading to implant loosening. Once an outburst of infection is controlled, short-lived neutrophils go into apoptosis and are removed by macrophages, whereas local B cells and plasma cells, with potentially longer life-spans, remain.

Although this hypothesis arising from current observations is tempting, it is largely not supported by the existing literature. The few studies that have investigated the feasibility of lymphocyte infiltrates in histopathological detection of implant-related infection have reported sensitivities lower than those achieved with detection of neutrophil infiltrates (Athanasou et al 1995, Pandey et al 1999). Likewise, analysis of lymphocyte subpopulations in the preoperative joint fluid aspirates is not useful in detecting knee replacement-related infection (Niki et al 2006). Data in this field is, however, limited, and further studies are warranted to explore the possibility that analysis of lymphocyte subpopulations in either histological samples or aspirated preoperative joint fluid might be useful in diagnosis of low-grade implant-related infection.

9.1.2. TLR expression and cell localization in aseptic interface tissue

We analyzed the presence and cell distribution of different TLRs in control synovial membranes and in aseptic and septic interface tissues, by use of antibodies specific for these different TLRs. Additionally, we evaluated TLR expression in control synovial membranes and in aseptic interface tissues with qRT-PCR. In control synovial tissues, TLR immunoreactivity was concentrated on some of the synovial lining cells, likely representing type A macrophage-like lining cells, vascular endothelium, and on occasional resident macrophages and fibroblasts in tissue stroma. Osteoarthritic synovial tissue is thus well equipped to recognize and react to possible foreign pathogens. Furthermore, TLR presence, especially RNA- and DNA-recognizing TLR3, TLR7, and TLR8 in synovial membrane, might also be relevant to the pathogenesis of autoimmune arthritis. In septic synovial tissues, macrophages and neutrophils accounted for most of the TLR immunoreactivity observed.

In comparison to control synovial tissues, the number of TLR immunoreactive cells was roughly doubled in aseptic interface tissues. The most apparent reason for this was the considerable macrophage infiltrates that accounted for most of the TLR immunoreactivity in the aseptic interface tissues. Despite the obviously increased number of TLR immunoreactive cells in the aseptic interface, the difference in number of TLR^+ cells did not reach statistical significance, likely due to the small number of samples investigated. However, in qRT-PCR analysis, utilizing a larger sample set, all TLRs, excluding TLR3 and TLR7, were significantly up-regulated in aseptic interface tissues when compared to their corresponding expression levels in control synovial membrane.

The sample set for the qRT-PCR analysis was validated by quantifying the expression levels of several chemokines previously linked to the pathogenesis of aseptic loosening (Goodman 2010). Increased production of the chemokines IL-8, CCL2, CCL3, and to a lesser extent, CCL4 in aseptic interface tissue has been reported in several earlier studies and is further confirmed by ours. These chemokines are also typically observed products of macrophages stimulated by various types of particles in vitro. Somewhat surprisingly, RANKL expression levels in the aseptic interface were low and did not differ from control synovial membrane levels, whereas OPG was significantly down-regulated in aseptic interface tissues. Although these findings contradict some previous findings of elevated RANKL levels and unchanged OPG levels, the resulting RANKL/OPG ratio still would seem to favor osteoclastogenesis. Similar results have also been reported (Koulouvaris et al 2008) Accordingly, the expression of osteoclast markers tartrate-resistant acidic phosphatase (TRAP) and catepsin K (CTSK) was strongly elevated in aseptic interface tissues, indicating active osteoclast formation in aseptic interface tissue.

Increased expression of TLRs in the interface tissue and their localization to infiltrating macrophages does not by any means convincingly show that TLRs mediate wear-particle-induced macrophage activation. This, however, at least indicates that infiltrating macrophages are expressing and even actively producing most of the TLRs; it suggests that these macrophages are well capable of recognizing danger signals in the interface tissue. Considering that some of the wear particles' inflammatory properties have been attributed to the bacterial structural components adhering to particle surfaces, and that TLRs generally mediate the recognition of such bacterial products, it seems likely that TLRs are involved in the recognition of PAMP-opsonized wear particles and in subsequent macrophage activation.

In this regard, especially noteworthy is the increased expression of TLR1, TLR2, TLR4, TLR6, and TLR9 in the interface tissues. These receptors recognize PAMPs that may most likely occur in the interface tissue including LPS (TLR4), gram-positive bacterial biofilms (TLR2/1, TLR2/6, TLR2, TLR9), and other gram-positive bacterial products possibly finding a route into interface tissue via the circulation. Endogenous TLR2 and TLR4 ligands have been identified, and thus one possibility is that these alamins as well—released by local cell necrosis and extracellular matrix fragmentation—may adhere to wear-particle surfaces, mediating particle recognition and a subsequent macrophage activation.

The significance of increased production of TLR5 and TLR8 in interface tissue remains undetermined, but might, like the active upregulation of other TLRs, indicate a shift in macrophage polarization towards the M1 phenotype. Interestingly, the two TLRs not up-regulated in aseptic interface tissue, TLR3 and TLR7, recognize viral-derived ssRNA structures and thus probably do not participate in recognition of wear particles. The apparent discrepancy between the immunohistochemical staining and qRT-PCR results in the expression of these two TLRs might result from the fact that macrophages

express some basal level of these two TLRs, as indicated by immunohistochemistry, but they are not induced to produce them further, as indicated by the qRT-PCR.

Direct, functional evidence that TLRs are involved in wear-particle recognition is emerging. Maitra et al (2008) showed that modified alkane polymers, released from UHMWPE and oxidized by interface tissue cells, can directly and specifically bind to TLR2 and TLR2/1 dimers and activate pro-inflammatory signaling as indicated by activation of NF-κB. Schmidt et al (2010) demonstrated that nickel ions are directly recognized by TLR4. Similarly, a 2012 report by Tyson-Capper et al shows that clinically relevant concentrations of cobalt ions, commonly released from metal-on-metal implants, can directly bind to and activate TLR4 signaling. Together these studies demonstrate that inorganic materials can, in some instances, be directly recognized by TLRs.

Pearl et al (2011) showed that knockout mice lacking the TLR adaptor protein MyD88 were highly resistant to PMMA particle-induced osteolysis and experienced no bone loss, whereas wild-type mice exhibited a roughly 15% decrease in local bone volume during a 10-day follow-up. Likewise, in macrophages isolated from the bone marrow of these mice lacking MyD88, PMMA particle-induced TNFα production was half the TNFα production of PMMA-particle-stimulated macrophages isolated from wild-type mice. Similarly, reduced TNFα production in particle-stimulated RAW 264.7 cells occurred if an MyD88-specific inhibitor was added to the culture system. Furthermore, at least during the 12-hour culture system, TNFα production was not dependent on the existence of TLR-adaptor TRIF; macrophages from TRIF knockout mice responded to particle stimulus with actually increased TNFα production, possibly due to compensatory up-regulation of TLRs and their intracellular signaling molecules in these knockouts.

The results of Pearl et al suggest that the MyD88-dependent pathway and associated TLRs are directly involved in the pro-inflammatory responses elicited by PMMA particles, and that at least in this relatively short-duration culture system, the TRIF-dependent pathway does not seem to play a role. The in vitro results were unaffected by PMMA-particle LPS contamination, because similar results occurred with Polymyxin B, an effective LPS-binding agent. However, the in vitro culture system apparently contained proteins (10% FBS); and naturally in the mouse model of particle-induced osteolysis used, a plentitude of potential DAMPs, both endo- and exogenous, are present and available to bind to particle surfaces and mediate their recognition. What thus cannot be concluded is that TLRs bind to and recognize PMMA particles directly without any DAMP involvement. Moreover, although TNFα production in particle-stimulated MyD88-deficient macrophages was reduced to half, these macrophages still produced considerable amounts of TNFα, indicating that some other mechanisms are still able to mediate particle recognition. Furthermore, we must note that disrupting MyD88 not only affects the TLR signaling but also impairs the IL-1 signaling pathway (O'Neill 2008). This adds some uncertainty to the results of the Pearl study, as IL-1β is an essential pro-inflammatory mediator of particle-induced inflammation; for example in caspase-1 knock-out mice, genetically unable to produce IL-1β, osteolysis is reduced in a mouse calvarian model of particle-induced inflammation (Burton et al 2012). On the other hand, these results by the Burton group also must be interpreted cautiously, because caspase 1 is required for the activation of MAL and is thus required for the activation of pro-inflammatory signaling via TLR2 and TLR4 (Miggin et al 2007).

Greenfield et al (2010) studied the role of TLR2 and TLR4 in the recognition and osteolysis caused by bacterial product-contaminated titanium particles. Using a mouse calvarial model of particle-induced osteolysis similar to that of Pearl et al, and $TLR2^{-/-}$, $TLR4^{-/-}$, and $TLR2^{-/-}/TLR4^{-/-}$ knockouts, as well as wild-type control mice, the Greenfield

group showed that the magnitude of osteolysis caused by titanium particles was about 5% greater at day 7 if particles are contaminated with PAMPs (either with LTA or LPS) and that this effect is dependent on the existence of their corresponding receptors TLR2 or TLR4. Concurrent results came from their in vitro experiments in which bone marrow macrophages isolated from these mouse strains and challenged with titanium particles up-regulated TNFα mRNA expression if LTA- or LPS-contaminated particles and the corresponding receptors TLR2 and TLR4 were also present on the macrophages. In contrast to results by Pearl et al, LTA- or LPS-free particles caused no detectable TNFα mRNA production. Interestingly, however, the amount of osteolysis caused by titanium particles that were not contaminated with LTA or LPS was similar in both wild-type mice and TLR knockouts, demonstrating that the recognition of, and osteolysis caused by, titanium particles without bacterial contamination is not mediated by TLR2 or TLR4. This somewhat contradicts the findings of the Pearl group. Furthermore, although bacterial-product contamination raised osteolysis by about 5 percentage points, titanium particles without contamination also caused a clear osteolytic reaction.

Although fascinating, the study by Greenfield et al reflects the shortcomings of the PAMP hypothesis of wear-particle recognition. The majority of the studies supporting the role of bacterial products in wear-particle recognition have followed the logic that wear particles with bacterial products cause more osteolysis in vivo, or more release of inflammatory mediators from macrophages in vitro, than do wear particles without bacterial contaminants (Ragab et al 1999, Daniels et al 2000, Bi et al 2001a, 2001b, 2002, Cho et al 2002, Brooks et al 2002). However, as LPS and also LTA are some of the most potent activators of the macrophages yet known, it seems somewhat obvious that particles contaminated with these products induce stronger reactions than do particles without them. Furhermore, wear particles of various natures clearly have inflammatory and osteolytic properties that are independent of PAMP, and perhaps also alarmin binding. The fundamental question is thus to what extent are these PAMPs present in the aseptic interface tissue? As PAMPs seem to be present in the interface tissues in at least some instances, it seems likely that at least in such cases, TLR-mediated wear-particle recognition and macrophage activation seems likely.

The discrepancy between the studies of Pearl et al indicating that MyD88 adapter is required for full, bacterial-product-free, PMMA particle-induced responses and Greenfield et al's indicating that TLR2 (and thus probably also TLR2/1 and TLR2/6 receptor dimmers) or TLR4 are not required for full, bacterial-product-free, titanium-particle-induced responses is intriguing. This discrepancy could have many explanations starting from the different material of the particles. An observation similar in both studies, mainly that particle-induced osteolytic responses were reduced but not completely abolished by disruption in TLR signaling, is, however, perhaps even more noteworthy. It thus seems that wear particles as such have some TLR-signaling-independent inflammatory properties which are further enhanced if exo- or endogenous DAMPs bind to the particle surface. This is likely the case in all in vivo systems, in which implanted biomaterials are immediately covered by a layer of host proteins and may also accumulate foreign bacterial products. We thus concluded that it is possible that TLRs are involved in wear-particle recognition, either by directly binding to the particles' polymeric surface, but probably more often via recognition of either exo- or endogenous DAMP molecules adhering to the particles' surfaces. The TLRs most clearly implicated in this process are TLR2 and TLR2/1, TLR2/6 dimers, and TLR9 as well as TLR4. The expression of all these receptors was increased in the interface tissue, and they localized to macrophage infiltrates.

9.2. TLR regulation as a response to titanium-particle stimulus (III)

TLR regulation as a response to titanium-particle stimulus we investigated both in vivo, with a mouse intramedullary model of particle-induced inflammation, and in vitro, with a mouse monocyte/macrophage cell line challenged with titanium particles.

The results of the in vivo experiment were somewhat surprising and their meaning difficult to interpret. On the control side, the number of TLR immunoreactive cells declined between the second and tenth week, likely reflecting physiological resolution of the surgical trauma-caused inflammatory reaction and possibly even reflecting the associated shift in the local macrophage activation state from the pro-inflammatory M1 phenotype towards the M2 phenotype, and subsequent reduction in TLR numbers.

Surprisingly, the number of TLR immunoreactive cells was generally reduced on the side that received titanium particles, a phenomenon observed both two and ten weeks post-operatively. The reason for this TLR down-regulation in the particle group remains somewhat elusive. One possibility is that the initial, strong inflammatory reaction caused by titanium particles leads to rapid down-regulation of the TLR system in order to limit the collateral damage from unrestrained inflammation. Indeed, studies using the same model system have reported a clear inflammatory reaction at two weeks after implantation on the particle side with increased production of CCL2, IL-6, and M-CSF (Warme et al 2004). Moreover, a periprosthetic membrane composed of fibroblasts and macrophages developed in about half of the cases that received titanium particles. Development of bone erosions also occurred, as well as bone remodeling. It is thus possible that the rapid down-regulation of the TLR system on the particle side was due to an initially stronger inflammation reaction.

Still, results of the in vivo experiment are somewhat difficult to interpret. As shown here, the aseptic interface tissue and foreign body reaction is characterized by an increase in the number of TLR-positive cells and evident up-regulation of TLR expression rather than by a reduction in the number of TLR-positive cells or TLR down-regulation. One possible explanation for this discrepancy between the model system and the patient samples stems fom the limitations of the model system. In contrast to the clinical situation where a continuous load of wear particles is released in the interface tissues, the titanium particles in the mouse model were delivered in single application. Using cultured human macrophages challenged in vitro with a single application of PMMA or titanium particles for nine days, Koulouvaris et al (2008) have shown that the expression of one possible M2 macrophage marker, chitinase 1, was up-regulated during the 9-day follow up, and concluded that long-term particle exposure would cause M2 rather than M1 macrophage polarization. Although these results are hardly conclusive, one possibility is thus that in the current experimental setting, also, one using single-dose and long-term particle exposure, particles had been, after a short inflammatory reaction, effectively contained in endosomes of macrophages that had assumed an M2-like phenotype and that this was reflected in the generally reduced TLR levels.

One additional possibity for this discrepancy in TLR regulation between the clinical setting and the mouse model system is that the reaction to the pure titanium particles might differ considerably from the UHMWPE or PMMA particles that are most prevalent in the clinical setting. Indeed, an otherwise similar mouse model system, but one utilizing UHMWPE particles instead of titanium particles, found a more clear-cut formation of macrophage infiltrates and giant cells as well as bone erosion, possibly resembling more closely the situation in human interface tissue (Epstein et al 2005).

In our macrophage culture system, the LPS-free titanium particles showed no effect on TLR mRNA expression during the 24-hour particle stimulation. Few studies have investigated TLR regulation in macrophages challenged with wear particles. Using cultured rat bone marrow macrophages, Takagi et al (2007) have shown that TLR4 and TLR9 mRNA levels were significantly down-regulated to about one-third their initial expression level after 3 hours of titanium-particle stimulation. Testing for possible LPS contamination of the particles used in the study by Takagi et al was not reported, so what cannot be definitively concluded is whether the TLR down-regulation observed was due to the effect of titanium particles alone. Indeed, using a similar rat bone-marrow-derived macrophage culture system. Hirayama et al (2011) showed that if titanium particles contained LPS, TLR4 and TLR9 mRNA levels were down-regulated more rapidly, although pure titanium particles seemed also to cause an eventual decline in the levels of these TLRs during 12-hour follow-up. In contrast, TLR2 mRNA levels were up-regulated and TLR5 levels unaffected by stimulation with LPS-containing particles. In experiments by Pearl et al (2011), PMMA-particle stimulus was associated with relative down-regulation of all TLRs, and of some of their intracellular signaling mediators in $MyD88^{-/-}$ knockout mice- derived macrophages, whereas in $TIRF^{-/-}$ knockout macrophages, this effect was abolished.

The wider meaning of these in vitro macrophage stimulation results remains to be determined. Based on current knowledge, we conclude that at least in the in vitro setting, wear-particle stimulus seems to cause only subtle changes in TLR levels and perhaps preferably down-regulation rather than up-regulation of TLRs, especially if particles are contaminated with bacterial products. This phenomenon is consistent with the negative-feedback loops typical of TLR signaling and is probably altered by factors inducing the full M1 macrophage phenotype. It can thus be further concluded that, at least based on results obtained from TLR regulation studies, particles without bacterial-product contamination cause only minimal activation of the TLR system, because the negative feedback mechanisms are not engaged by LPS-free particles. What thus seems likely is that what is responsible for the up-regulation of TLR in interface tissues is other factors and signals derived from the peri-implant microenvironment, rather than any direct effect of the wear particles or contaminating PAMPs.

9.3. Effect of macrophage polarization on wear-particle responses (IV)

To assess the effect of macrophage polarization on macrophage wear-particle responses, we produced M0, M1, and M2 macrophages and stimulated them with LPS-free titanium particles. These macrophages' response to stimulation was evaluated and compared by genome-wide microarray analysis, qRT-PCR, and a protein suspension array. Gene ontology and signaling pathway analyses allowed further analysis of the possible biological significance of the gene- expression changes. Furthermore, macrophage-polarization-induced changes in cell morphology, motility, and phagocytotic activity we evaluated by means of time-lapse phase contrast microscopy.

We found that macrophage wear-particle responses were greatly influenced by macrophage polarization state, because each of the macrophage types responded to wear-particle stimulus with a unique profile of transcriptome and proteome changes. In comparison to M0 macrophages, the overall inflammatory and chemotactic response to wear particles was enhanced in M1 macrophages, as indicated by GO and SPIA as well as by detailed examination of the trascriptome and proteome changes.

In addition to production of cytokines (TNFα, IL-1β, IL-6), chemokines (IL-8, CCL3) and growth factors (GM-CSF) previously recognized as playing a role in the pathogenesis of aseptic loosening, our wide screening methods made it possible to identify several novel, potentially osteolytic cytokine mediators from M1 and to a lesser extent M0 wear-particle response. These factors, including OSM, TNFSF9, TNFSF14, CXCL2, CCL20, CCL4, CCL7, IL-7, sCD40L, FLT 3 ligand, G-CSF, and FGF-2 support osteoclast formation or function in other settings but have not yet been linked to the foreign-body response (Lean et al 2001, Abe et al 2002, Palmqvist et al 2002, Shimoaka et al 2002, Nakano et al 2004, Yu et al 2004, Hui et al 2005, Lee S et al 2005, Edwards et al 2006, Lee H et al 2006, Hirbe et al 2007, Lisignoli et al 2007, Yang et al 2008, Ishida et al 2009, Ha et al 2010, Walker et al 2010, Guihard et al 2012) These mediators are thus interesting topics for further studies and novel targets for therapeutic interventions.

In contrast, all these wear-particle responses were effectively suppressed in M2 macrophages, which seemed to respond to particle stimulus with only sporadic trascriptome changes that formed no functional groups. Still, the M2 macrophages actively moved around the culture dish and effectively phagocytosed titanium particles; the conclusion thus is that M2 macrophages effectively constrained wear particles into intracellular compartments without any inflammation reaction.

A similar phenomenon emerged from Trindade et al (1999a, 1999b), who showed that IFNγ pre-treatment enhances the production of TNFα and IL-6 from human monocytes stimulated with PMMA particles and that IL-4 pre-treatment suppresses the production of TNFα, IL-1β, and GM-CSF from PMMA-particle-stimulated human monocytes when compared to non-treated monocytes. Im and Han (2001) showed a similar suppressive effect of IL-4 pre-treatment on production of TNFα and IL-6 from human monocytes stimulated with titanium-alloy particles. Likewise, a very recent study by Rao et al (2012) demonstrated that IL-4 administration even after the particle stimulus reduces production of TNFα from isolated mouse bone marrow macrophages stimulated with PMMA particles with or without LPS. This phenomenon was more clear-cut in the PMMA+LPS group, likely due to a more prominent inflammatory reaction due to LPS presence, but reduction in production of TNFα occurred in both groups. Additionally, the results of Rao et al demonstrate that sequential modulation of a macrophage phenotype, from the LPS-induced M1-like phenotype to the IL-4-induced M2-like phenotype, successfully reduces PMMA particle-induced TNFα production.

In addition to IL-4, also IL-10 and IL-1ra can reduce wear-particle-induced inflammatory responses. Both Pollice et al (1998) and Trindade at al. (2001) demonstrated the IL-10s' ability to diminish TNFα and IL-6 production from PMMA- and titanium particle-stimulated cultured human monocytes; Im and Han (2001) demonstrated a similar phenomenon by using titanium alloy particles. Yang et al (2004) demonstrated—in a mouse air-pouch model of particle-induced osteolysis—the ability of both IL-10 and IL-1ra gene transfer to effectively mitigate UHMWPE particle-induced inflammation and the accompanied osteolysis.

These studies, consistent with the current results, provide clear evidence of the principle that modulation of the macrophage activation state in the interface tissue, either with biomaterial solutions or pharmacologically, might serve as a useful means to reduce the inflammatory and osteolytic reaction resulting from unavoidably forming wear particles. Similarly, results suggest that, at least in a cell-culture setting, wear particles cause only limited macrophage activation, and that their pro-inflammatory properties are at least partially dependent on prevailing micro-environmental conditions. Here, the phenomenon was modeled with IFNγ, but the principle is possibly expandable to

encompass other M1-inducing factors that might be present in the interface tissue such as bacterial biofilms or exo- or endogenous danger signal molecules adhering to wear-particle surfaces. Indeed, LPS typically used as a model of wear-particle-adhering PAMP causes, via recognition by TLR4 and the subsequent TIRF-dependent signaling pathway, the production of type-1 interferons and thus an M1-like macrophage phenotype via auto- and paracrine signaling. Additionally, balance of M-CSF and GM-CSF, which induce M2 and M1-like macrophage phenotypes, might be a similarly important factor in determining macrophages' wear-particle responses. The extent to which wear particles cause inflammation and osteolysis may thus be dependent on the local balance of M1- and M2-inducing factors; modulation of this balance might provide a novel target for therapeutic interventions.

An interesting hypothesis raised by the current results is that in addition to local micro-environmental factors, systemic factors such as chronic low-grade systemic inflammation and the accompanying chronic production of inflammatory cytokines, associated with atherosclerosis, metabolic syndrome, and periodontitis, may be important in determining the M1/M2 balance of macrophages in the interface tissue, and thus in the mode by which the host reacts to the inevitably forming wear particles (Pischon et al 2007, Rocha et al 2009, Gregor et al 2011). Indeed, Gordon et al (2010) have demonstrated that peripheral blood monocytes isolated from subjects with previous aseptic THR loosening isplayed increased production of several inflammatory cytokines and thus a more M1-like phenotype in response to LPS stimulation than did monocytes isolated from subjects developing no hip replacement loosening. Gordon et al conclude that the innate immune system of patients experiencing aseptic THR loosening is genetically more reactive. An equally plausible explanation is that these results reflect the underlying inflammatory status of the system and a correspondingly increased macrophage-activation state. Yet another possibility is that the inflammation at a systemic level or in interface tissues leads to an increased proportion of circulating inflammatory $CD14^{+}CD16^{+}$ monocytes that, once migrated into interface tissue, preferably differentiate into inflammatory M1 macrophages. This systemic inflammation may be one answer as to why only a relatively small proportion of THR recipients eventually develop aseptic loosening. Similarly, controlling the systemic inflammation might be one means to limit wear-particle-induced osteolysis. Indeed, HMG-CoA reductase inhibitors (statins) that, in addition to their intended effects on cholesterol metabolism have pleiotropic anti-inflammatory properties, can reduce macrophage activation and wear-particle-induced osteolysis both in model systems and in clinical trials (von Knoch et al 2005, Laing et al 2008, Thillemann et al 2010).

These phenomena may also provide some insight into the mechanisms of wear-particle recognition and induced inflammation. SPIA analysis suggests that TLR- or NOD-like receptor signaling is involved in the wear-particle response of M1 macrophages. This must be interpreted cautiously, however, as only the end products (including inflammatory cytokines and chemokines due to NF-κB and AP-1 activation) of these receptor-signaling pathways were enriched into GO and SPIA analyses. It is thus quite possible that activation of these transcription factors is mediated by some other, perhaps unknown, particle-recognition pathway. On the other hand, the up-regulation of the TLR system in M1 macrophages may well be one factor that enhances these cells' wear-particle responses, although other IFNγ effects, such as complex and various synergistic interactions between STAT1 and NF-κB pathways, or suppressed production of IL-10, may also explain the increased responsiveness of M1 macrophages to wear particles (Schroder et al 2006).

Assuming TLR involvement in wear-particle recognition, it is then interesting that wear-particle stimulation did elevate production of pro-inflammatory chemokines and cytokines; this suggests the activation of the MyD88-dependent signaling pathway and associated TLRs, culminating in the activation of AP-1 and NF-κB. In contrast, wear particle stimulus led neither to production of IFN-α2, nor to production of any other type-I interferon, thus excluding the TIRF-dependent pathway culminating in the activation of IRF3.

In addition to these TLR-signaling pathways, the wear-particle-induced production of IL-1β suggests inflammasome activation, perhaps via particle-induced endosomal damage and cell stress as described previously (Maitra et al 2009, Burton et al 2012). One possibility is thus that, in addition to, or instead of TLR signaling, wear-particle-induced NF-κB activation is initially mediated via endosome damage, subsequent inflammasome activation, and the following auto- and paracrine IL-1β signaling.

Suppression of the inflammatory response in M2 macrophages is likely mediated by general suppression of the TLR signaling and inflammatory response in these cells via inhibition of NF-κB and STAT signaling by the up-regulation and action of PPARγ. M2 macrophages also effectively produced IL-10 and IL-1ra that likely further inhibited their wear-particle-induced responses.

10. Summary and conclusions

During the previous decade, the strict dichotomy between the two major complications of otherwise very successful THR surgery, namely septic and aseptic hip replacement loosening, has been increasingly questioned. For instance, subclinical bacterial biofilms occur in at least in some cases of apparently aseptic implant loosening. Likewise, pro-inflammatory and osteolytic properties of wear particles depend at least partially on the presence of bacterial components adhering to their surfaces. These observations lead to the hypothesis that recognition of bacterial-product-coated wear particles and subsequent activation of interface tissue macrophages into the inflammatory phenotype might be mediated by macrophages' TLRs. Thus, the extent to which the various TLRs are present and how they localize in the aseptic and septic interface tissues called for evaluation. In addition, we evaluated the direct effect of wear particles on TLR levels in an animal model of wear-particle-induced inflammation and in a macrophage culture system. Moreover, in hopes of identifying cell populations potentially useful as diagnostic markers of subclinical implant-related infection, we evaluated cell populations present in typical septic and aseptic interface tissue. In addition, as immunological research indicates that the expression of TLRs and macrophages ability to produce inflammatory mediators is largely dependent on macrophage polarization, we further hypothesized that macrophage polarization might be an important determinant in the way that macrophages react to wear particles.

The main conclusions of the study are:

1) Macrophages of aseptic interface tissue express TLR1-9 and actively produce all of these TLRs except TLR3 and TLR7. Based on the literature, what seems likely is that TLRs are involved in wear-particle recognition and subsequent macrophage activation. This may occur either by direct recognition of the particle's polymeric surface or of metal ions released from the implant, but probably more often occurs via recognition of either exo- or endogenous DAMP molecules adhering to the particle surface. The TLRs most clearly implied are the ones recognizing bacterial and endogenous DAMP molecules, namely TLR2 and TLR2/1, TLR2/6 dimers, and TLR9, as well as TLR4.

2) Comparison of inflammatory cell populations present in septic and aseptic interface tissues revealed that, in addition to neutrophils, B lymphocytes and plasma cells might be useful marker cells in the diagnosis of low-grade implant-related infection. Further studies are warranted to explore the possibility that analysis of lymphocyte subpopulations in either intraoperative histological samples or preoperative joint fluid aspirates might be a useful diagnostic tool for low-grade implant-related infection

3) In a mouse model of particle-induced inflammation, wear particles led to down-regulation rather than to up-regulation of TLR levels. They had no effect on TLR mRNA levels in a mouse macrophage culture system. The up-regulation of TLRs in aseptic interface tissue is thus likely mediated by factors other than the direct effect of wear particles on macrophages.

4) Macrophage polarization has a clear impact on macrophage wear-particle responses. In comparison to M0 macrophages, the inflammatory and chemotactic wear- particle response was enhanced in M1 macrophages but was effectively suppressed in M2 macrophages which effectively contained particles in their intracellular compartments. Results suggest that in addition to wear-particle characteristics and DAMPs adhering to particle surfaces, the local cytokine milieu also determines the extent to which macrophages are activated by wear particles. This effect may be mediated by differing expression and regulation of TLRs or their signaling mechanisms among macrophage subtypes. The cytokine profile secreted by M1 macrophages implied a MyD88-dependent TLR-signaling pathway, suggesting the role of TLR2, TLR2/1, TLR2/6, or TLR9, rather than TLR4. The production of IL-1β implied inflammasome activation. A summary of the proposed mechanisms of aseptic ostelysis is presented in Figure 9.

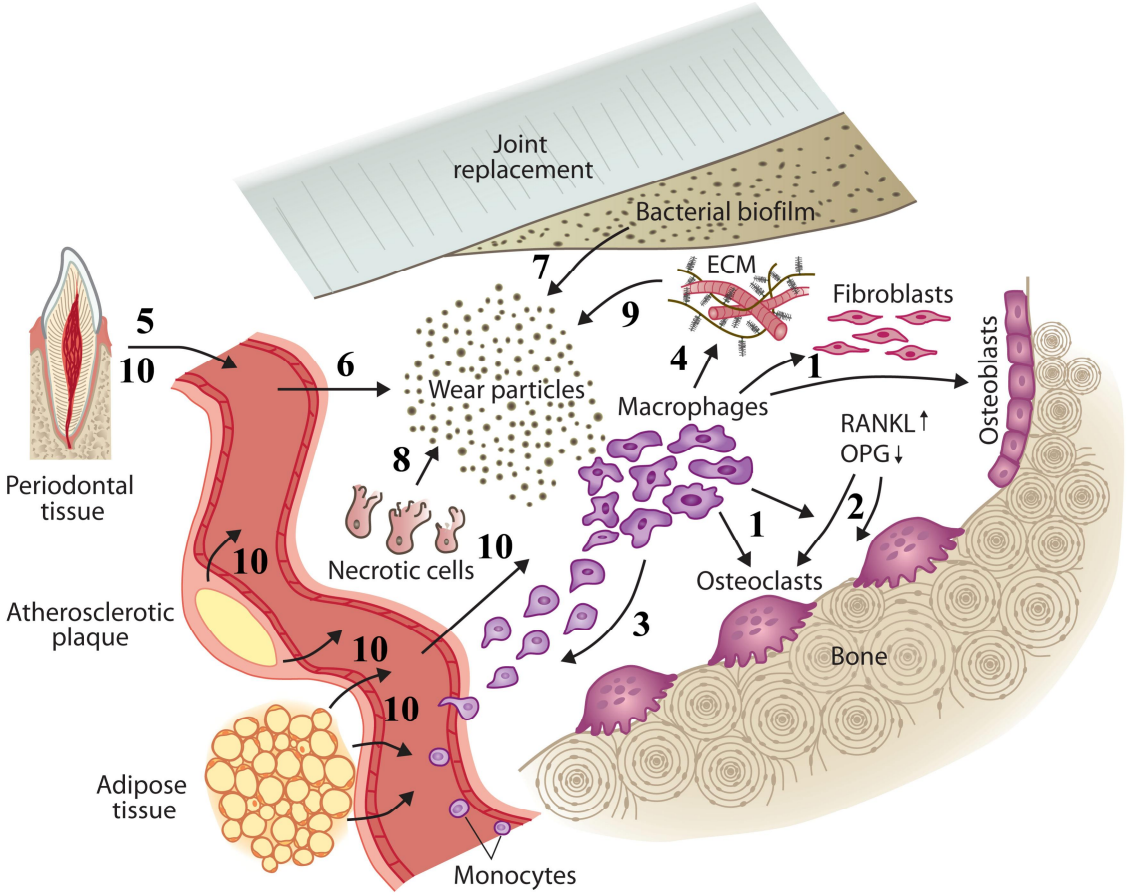

Figure 9. Proposed mechanisms of aseptic osteolysis. Wear particles are generated due to abrasion between THR components. Pressure waves of the pseudosynovial fluid effectively distribute these particles into surrounding interface tissue where they are phagocytosed by macrophages. Macrophages are activated to produce (1) pro-inflammatory cytokines which directly enhance osteoclast formation and function. Furthermore, macrophage-derived inflammatory mediators enhance production of RANKL and suppress production of OPG from local fibroblasts and osteoblasts, so that the local RANKL/OPG ratio increases, favoring osteoclast formation and active bone resorption (2). Wear-particle-activated macrophages also produce (3) chemokines that recruit additional monocytes into interface tissue and (4) MMPs that degrade the local ECM. The extent to which wear particles cause macrophage activation appears to be dependent not only on wear-particle characteristics or DAMP molecules adhering to

particle surfaces but also on the local cytokine milieu determining the macrophage polarization state. PAMPs are released into the circulation from body surfaces, for example from chronic inflammation in periodontal tissue (5), and these hydrophobic molecules accumulate on wear particles, owing to their great surface area (6). Additional possible PAMP sources include subclinical bacterial colonization of the implant surface (7). Futhermore, endogenous alarmins released due to cell necrosis (8) and ECM damage (9) may adhere to wear-particle surfaces. DAMP-coated wear particles are then recognized by macrophages via TLRs. TLR signaling causes macrophage activation and possibly M1-type macrophage polarization and production of pro-inflammatory cytokines, ultimately leading to osteoclastogenesis. Additionally, wear-particels can induce production of GM-CSF that causes further M1-type macrophage activation. Limiting the action of M1-polarizing factors and perhaps also promoting M2 macrophage polarization may be a means to control this sequence of events. The emerging hypothesis that low-grade inflammation at systemic level, and the accompanying release of low levels of inflammatory cytokines from adipose tissue (10), atherosclerotic plaques (10), or periodontal tissue (10) may be a determinant for the general systemic M1-M2 balance of macrophages, or possibly for the levels of circulating monocyte subpopulations. Therefor they may influence the individual mode by which macrophages react to wear particles, a process that warrants further investigation. THR - total hip replacement; RANKL - receptor activator of nuclear factor kappa B ligand; OPG - osteoprotegerin; ECM - extracellular matrix; MMP - matrix metalloproteinase; DAMP - danger-associated molecular pattern; PAMP - pathogen-associated molecular pattern; TLR - toll-like receptor; GM-CSF - granulocyte-macrophage colony-stimulating factor. Illustration by Helena Schmidt.

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